THROUGH SCIENCE WE DEFEND

TECHNICAL REPORT
NATICK/TR-02/025

AD_____

SENSITIVITY OF SPORES TO HYDROSTATIC PRESSURE: MECHANISMS OF ACTIVATION, INJURY AND REPAIR

PHASE 1

by
Dallas G. Hoover*
Cynthia M. Stewart*
Anthony Sikes**
and
C. P. Dunne**

***University of Delaware**
Newark, DE 19717-1303

September 2002

Final Report
October 1995 - February 1997

Prepared for
****U.S. Army Soldier and Biological Chemical Command**
Soldier Systems Center
Natick, Massachusetts 01760-5018

DISTRIBUTION STATEMENT A
Approved for Public Release
Distribution Unlimited

20021023 032

DISCLAIMERS

The findings contained in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Citation of trade names in this report does not constitute an official endorsement or approval of the use of such items.

DESTRUCTION NOTICE

For Classified Documents:

Follow the procedures in DoD 5200.22-M, Industrial Security Manual, Section II-19 or DoD 5200.1-R, Information Security Program Regulation, Chapter IX.

For Unclassified/Limited Distribution Documents:

Destroy by any method that prevents disclosure of contents or reconstruction of the document.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.
PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
30 SEPT. 2002	FINAL	OCT 1995 - FEB 1997

4. TITLE AND SUBTITLE
SENSITIVITY OF SPORES TO HYDROSTATIC PRESSURE: MECHANISMS OF ACTIVATION, INJURY AND REPAIR - PHASE 1

5a. CONTRACT NUMBER
DAAK60-95-C-2060

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dallas G. Hoover*, Cynthia M. Stewart*, Anthony Sikes**, and C.P. Dunne**

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
*Department of Animal and Food Science
035 Townsend Hall
University of Delaware
Newark, DE 19717-1303

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
**Soldier Systems Center
U.S. Army Soldier and Biological Chemical Command
Natick MA 01760-5018

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
NATICK/TR-02/025

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for Public Release, Distribution is unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The sensitivity of spores *of Bacillus subtilis* 168 *and Clostridium sporogenes* PA3679 to the effects of pressure (4,000 atmospheres) alone and in combination with nisin, temperature and pH were determined. The results showed that PA3679 spores were more resistant to nisin than *B. subtilis* 168 but were more sensitive to the combined effects of pressure, elevated temp. and reduced pH. Pressure oscillations of spores' suspensions did not result in a significant $\log_{10}$ reduction in spore count as compared to continuous pressurization. When spores of *B. subtilis* 168 and PA3679 were examined for sensitivity to pressure (4,000 atm.) in different buffers at pH 7.0 in the presence of food-grade salts, no significant difference was observed, relative to buffers. Sodium acetate (up to 5%) had no adverse effect on the viability of spores of *B. subtilis* 168 and PA3679. However, *B. subtilis* 168 spores were less sensitivity to sodium nitrite (≤ 5% caused no inhibition) than spores of PA3679 (0.02% caused inhibition). When spores of *B. subtilis* 168 and PA3679 were tested for sensitive to sucrose laurate, pressure and temperature, PA3679 was highly sensitive at 25°C but *B. subtilis* was not. However, at 45°C spores of PA3679 were equally sensitive to the combo. Inoculated pack studies revealed that when the combination of pressure (4,000 atm.), temp (55°C) and nisin (0.1 IU/mL) were used spores count (300,000 spores/pouch) was reduced by 3 $\log_{10}$.

15. SUBJECT TERMS

SENSITIVITY	TEMPERATURE	MICROORGANISMS	FOOD PRESERVATION
SPORES	pH FACTOR	HYDROSTATIC PRESSURE	
BACILLUS SUBTILIS	FOOD PROCESSING	CLOSTRIDIUM SPOROGENES	

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			A. SIKES/ C.P. DUNNE
U	U	U	SAR	60	19b. TELEPHONE NUMBER *(Include area code)* (508)233-4383/233-5514

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

This study was conducted by Dallas G.Hoover and Cynthia Stewart, Department of Food Science, University of Delaware, Newark, DE, under the general supervision of Mssrs. C. Patrick Dunne, Project Manager and Anthony Sikes, contracting officer's representative (COR), who are both members of the Combat Feeding Group, Soldier Systems Center, Natick, MA.. This study was initiated on 2 September 95; it summarizes the results from Phase I of the project titled "Sensitivity of spores to hydrostatic pressure: Mechanisms of inactivation, injury, and repair", which ended 6 June 97.

This report examines the response of spores of non-pathogenic *Bacillus* and *Clostridium* species to high hydrostatic pressure alone, and in combination with other processing adjuncts to evaluate the mechanism of spore inactivation, injury and repair. The study was divided into two phases. In Phase I, strains of the test organisms were evaluated against temperature of pressure treatment, the presence of food preservatives, e.g., nisin and sodium nitrite, acidic growth conditions, and pressure pulsing.

Phase II examined the mechanism of pressure-induced inactivation and damage to spores of *Bacillus* and *Clostridium* in the presence of various processing aids, e.g., sucrose laurate.

This study was supported by the U.S. Army Soldier Systems Center Broad Agency Announcement Grant Award number DAAK60-95-C-2060

SUMMARY

Introductory work was completed for *Bacillus subtilis* 168 and PA 3679 (*Clostridium sporogenes*) regarding sensitivity to pressure exposure (4,000 atm) alone and in combination with nisin, temperature and pH. In comparison to *B. subtilis* 168, PA 3679 was more resistant to nisin, but proved to be more sensitive to the combined effects of exposure to 4,000 atm, elevated temperatures, and reduced pH. Approximately 1 million spores/mL of *B. subtilis* 168 were eliminated by exposure to 4,000 atm, 0.6 IU/mL nisin and 45°C at pH 5.0 for 15 min. For PA 3679, approximately 1 million spores/mL were inactivated by exposure to 4,000 atm and 70°C for 15 min at pH 4.0 without nisin present.

Five-minute pressure oscillations at 4,000 atm and 25°C did not result in a significant decrease in spore counts as compared to continuous pressurization. The same conditions of pressure treatment at 45°C showed that pressure oscillation as compared to a continuous pressurization will enhance spore inactivation to a demonstratable, but not substantial extent. The type of buffer for pressure treatment of spore suspensions was also shown to be a factor in pressure inactivation. At pH 5.0, different degrees of spore inactivation were seen with *B. subtilis* 168 and *C. sporogenes* PA 3679 at both 25° and 45°C with acetate, citrate, and citrate phosphate buffers. Although at the same pH, different ionic concentrations were represented in the three buffer systems. These spore counts have not yet been statistically examined, but it appears that the citrate phosphate buffer is more detrimental to spore outgrowth with pressurization, especially for *C. sporogenes* PA 3679. With spore suspensions of *B. subtilis* 168 it was found that 90°C at pH 4.0 for 30 min without pressure was enough to eliminate over 1×10^6 spores/mL, indicating that at such high treatment temperatures, low pH is a very significant factor that should be optimized.

Spore suspensions of *B. subtilis* 168 and *C. sporogenes* PA 3679 were examined for response to high hydrostatic pressure in different buffers at pH 7.0 and in different salts of food-grade preservative compounds. *B. subtilis* 168 and *C. sporogenes* PA 3679 displayed no difference to the different buffers (all at pH 7.0) with pressure treatment indicating that any difference in disassociation of the buffers was not a significant factor regarding spore inactivation by hydrostatic pressure under the conditions examined. *B. subtilis* 168 was reduced by about 2-$\log_{10}$ CFU/mL at 4,000 atm and 45°C while PA 3679 was not affected.

Both *B. subtilis* 168 and PA 3679 were resistant to sodium acetate levels up to 5.0% (with no decrease in CFU/mL). Spore suspensions of *B. subtilis* 168 were much more resistant to sodium nitrite (up to 5.0% with no effect on CFU/mL) than were spore suspensions of PA 3679 (inhibition occurred as low as 0.02% sodium nitrite). *B. subtilis* showed inhibition at 0.7% benzoic acid while PA 3679 did not show inhibition until a concentration of 2.0% benzoic acid was reached. For both strains, no synergy or additive effect was shown with combinations of pressure treatment and these preservative compounds. The only exception was noted with *B. subtilis* 168 and 0.5 to 1.0% sodium nitrite at 45°C and this effect was slight.

Spores suspensions of *B. subtilis* 168 and *C. sporogenes* PA 3679 were pressure-treated by Marcia Walker at Oregon State University because of the higher pressure magnitudes attainable in their pressure unit (at the time of sample receipt, 5,100 atm was the pressure maximum).
For either strain at 25°C, spore suspensions of 1×10^6/mL at pH 4.0 were inactivated by 30-min exposures to 5,100 atm; however, at pH 5.0 to 7.0 approximately 100 spores/mL of *B. subtilis* 168 remained. For PA 3679 about 1,000 spores/mL were recoverable at pH 5.0 or 6.0, and at pH 7.0 approximately 1×10^5 spores/mL remained. Spores of PA 3679 were found to be extremely susceptible to sucrose laurate. Spores of *B. subtilis* 168 were found resistant to sucrose laurate and no significant reductions (>1 $\log_{10}$) were evident at 25°C when pressurization and sucrose laurate were combined to eliminate spores of *B. subtilis*; however, dramatic reductions in spore populations were seen when pressure treatments were done at 45°C along with exposure to sucrose laurate. At pH 4.0 to 6.0, no colonies were detected from *B. subtilis* spore suspensions of 1×10^6/mL after pressurization at 45°C and exposure to sucrose laurate.

Inoculated pack studies with spores of *Clostridium sporogenes* PA3679 in sealed pouches containing reinforced clostridial medium showed that application of 4,000 atm for 15 min at 95°C was effective in inactivating 3×10^5 spores/pouch. Treatments using pressure (4,000 atm) alone, temperature (up to 95°C) alone and nisin alone, were ineffective. At a nisin concentration of 0.1 IU/mL, a minimum pressure-treatment temperature of 55°C was required to inactivate 3×10^3 spores/pouch.

SENSITIVITY OF SPORES TO HYDROSTATIC PRESSURE: MECHANISMS OF INACTIVATION, INJURY, AND REPAIR

PHASE 1

INTRODUCTION

Compared to vegetative forms of microorganisms, dormant spores of *Bacillus* and *Clostridium* are highly resistant to physical and chemical agents that include heat, drying, chemical disinfectants such as hydrogen peroxide, radiation, and pressure (Hoover *et al.*, 1989; Farkas, 1994; Setlow, 1994). In food processing, the use of heat as a sole preservative method for the destruction of spores in low-acid foods usually results in deleterious quality changes to nutritive value, color, flavor and aroma (Jay, 1986). High hydrostatic pressure as a means of food preservation was first examined by Hite (1899); however, in the last 15 years renewed interest in cold-pasteurization technologies has generated new applications for hydrostatic pressure in food processing. Hydrostatic pressure can be an effective means of reducing or eliminating microorganisms in food at pressures of 300-600 Mpa (Ogawa *et al.*, 1990; Shigehisa *et al.*, 1991; Mertens and Knorr, 1992; Tanaka and Hatanaka, 1992); however, bacterial spores and viruses have been shown to be highly resistant to high pressures, even at pressures of up to 1020 Mpa (Giddings *et al.*, 1929; Johnson and ZoBell, 1949; Timson and Short, 1965; Sale *et al.*, 1970). Studies have shown that the antimicrobial effect of high hydrostatic pressure can be increased when used in combination with heat, low pH, CO_2, bacteriocins such as nisin, and preservatives as sucrose laurate (Haas *et al.*, 1989; Mallidis and Drizou, 1991; Mertens and Knorr, 1992; Sikes and Whitfield, 1992; Kalchayanand *et al.*,

1994). Therefore, the hurdle concept (Leistner and Rodel, 1976), where a number of processes or barriers are used to ensure food safety and preservation, readily applies to the optimization of high hydrostatic pressure as a food processing method for low acid, neutral-pH foods given the resistances of bacterial endospores (Hoover *et al.*, 1989). A combination of moderate treatments, or barriers, would most likely lead to a food preservation method that would utilize high hydrostatic pressure and be effective against bacterial sp

If high hydrostatic pressure technology is to have a ores, while having less effect on food quality than a high temperature sterilization process. broad application for foods, its effects against bacterial endospores in low-acid foods must be better understood and optimized.

The overall objective of this project is to examine the response of spores of *Bacillus* and *Clostridium* to high hydrostatic pressure alone and in combination with other processing aids to evaluate the mechanisms of spore inactivation, injury and repair. Accompanying factors for evaluation and development with high hydrostatic pressure in this Phase I study (as described in experimental objectives of the proposal) include the temperature of pressure treatment, the presence of preservatives such as nisin and sodium nitrite, mild acidity, and the use of pressure pulsing. Sucrose laurate was also examined in combination with pressure treatment.

MATERIALS AND METHODS

Production of spore crops

Bacillus subtilis 168 was obtained from the Bacillus Genetic Stock Center (Columbus, Ohio). *Clostridium sporogenes* 7955 (PA 3679) was obtained from the American Type Culture Collection (Rockville, MD). Spores of *B. subtilis* 168 were prepared by spread-plating a 24-h nutrient broth culture onto 100 nutrient agar (NA) plates. Plates were incubated at 37°C. Sporulation was assessed by phase contrast microscopy and spores were harvested after 6 days with sterile distilled water and a bent-glass rod. Spores of PA 3679 were prepared by spread-plating a 24-h reinforced clostridial medium (RCM) culture onto 100 reinforced clostridial agar (RCA) plates. Plates were incubated in a Coy anaerobic chamber at 37°C. Sporulation was assessed by phase microscopy and spores were harvested after 6 days with sterile distilled water and a bent-glass rod. The spore crops were purified using methodology described by Nicholson and Setlow (1990). The crops were centrifuged and the supernatant was removed. The pellets were washed with 20 mL of 1 M KCl/0.5 M NaCl solution and centrifuged. The supernatant was removed and 10 mL of Tris.Cl (pH 7.2) containing 50 µg/mL lysozyme was added. The spore suspensions were incubated for one hour at 37°C. The spores were cleaned by alternate centrifugation and washing with 1.0 M NaCl; sterile distilled water; 0.05% sodium dodecyl sulfate (SDS); TEP buffer (50 mM Tris-Cl buffer, pH 7.2, with 10 mM EDTA and 2 mM phenylmethylsulfonylfluoride); 3 washes with sterile distilled water. The spore crops were stored in sterile water at 4°C until needed.

Sensitivity of spores of *B. subtilis* and PA3679 to nisin

Stock solutions of Nisaplin™ (1×10^6 IU/g; Aplin and Barrett, Ltd, Beaminster, Dorset, UK) with concentrations of 100 and 1000 IU nisin/mL were made in 0.02 M HCl, autoclaved, and stored at -60°C for up to one week until needed. The nisin activity of the stock solutions was checked by titering against *Lactococcus cremoris* 14365. The nisin stock solutions were aseptically added to sterile, tempered nutrient agar (NA + nisin) in concentrations from 0.2 to 1.0 IU/mL for the experiments with *B. subtilis* and added to sterile, tempered reinforced clostridial agar (RCA + nisin) in concentrations of 1.0 to 500 IU/mL for the experiments involving PA 3679. Aliquots were taken from the respective spore crops and diluted with sterile 0.1%-peptone water to an approximate concentration of 10^6 spores/mL. Each was serially diluted in 0.1%-peptone water and pour-plated with NA + nisin or RCA + nisin. Initial spore concentrations were determined in NA or RCA. The *B. subtilis* plates were incubated at 37°C for 24 h. The PA 3679 plates were incubated anaerobically at 37°C for 48 h.

Pressure treatments

The spores of *B. subtilis* and PA 3679 were subjected to various combinations of 4,000 atm pressure with: 25, 45, 70, 85 or 90°C; pH 4.0, 5.0, 6.0 or 7.0; and exposure times of 15 or 30 min, with plating after completion of pressurization cycle in the Autoclave Engineers model in NA or RCA with and without nisin. Spores were diluted with sterile McIlvaine's citrate phosphate buffer (pH 4.0-7.0) to approximately 1×10^6 spores/mL. A starting sample was taken to determine the initial concentration of each spore load. The spore suspensions were aseptically transferred to pouches, heat-sealed, and heat-shocked at 80°C for 10 min. The heat-shocked spores were immediately cooled in ice water and then exposed to the appropriate pressure, temperature, and time combinations. All samples treated above 25°C were immediately cooled in ice water after

treatment. The pouches were washed with antiseptic soap, aseptically cut open, and samples removed. The zero-time samples were serially diluted in sterile 0.1%-peptone water and pour-plated with tempered NA or RCA for *B. subtilis* and PA3679 samples, respectively. The treated samples were serially diluted and pour-plated with NA and NA + 0.6 IU/mL nisin, or RCA and RCA + 10.0 IU/mL nisin. The *B. subtilis* samples were incubated at 37°C for 24 h before counting and the PA 3679 samples were counted after anaerobic incubation at 37°C for 48 h.

Pressure oscillations

Spores of *B. subtilis* 168 were suspended to a concentration of 1 x 10^6/mL in 10 mL of McIlvaine's citrate-phosphate buffer (pH 4.0, 5.0, 6.0 or 7.0). Samples were placed into pouches and heat-sealed. Spore suspensions were heat-shocked at 80°C for 10 min and cooled in ice water before pressurization. The spore samples were pressurized at 4,000 atm at 25 or 45°C using multiple [5-min pressurization/depressurization: 5-min pressurization] increments. Samples were serially diluted, pour-plated with NA and incubated at 37°C.

Citrate and acetate buffers

Acetate buffer (0.2 M, pH 5.0) and citrate buffer (0.1 M; pH 5.0) were used to determine if different buffers would have an effect on the viability of spores with pressurization. Approximately 1 x 10^6 spores/mL of *B. subtilis* and *C. sporogenes* PA 3679 were suspended in 10 mL of acetate or citrate buffer, placed into pouches, heat-shocked, and pressurized at 4,000 atm and 25 or 45°C for 30 min. Samples pressurized at 45°C were cooled in ice water after pressurization. Samples and initial counts were serially diluted and pour-plated in NA (168) or reinforced clostridial agar (PA 3679). *B. subtilis* 168 was incubated at 37°C for 24 h. Strain PA 3679 was incubated for 48 h at 37°C in the Coy

anaerobic chamber.

Tris-HCl, *bis*-Tris-HCl and *bis*-Tris-propane buffers

Selected buffers were chosen according to their pressure-resistant characteristics (Funtenberger *et al.*, 1995). Tris-HCl buffers have demonstrated the ability to keep fairly constant pH values under high pressure. *Bis*-Tris and *bis*-Tris-propane buffers were selected because each retains better buffering capacity than Tris-HCl at neutral pH.

Spores of *B. subtilis* 168 and *C. sporogenes* PA 3679 were suspended to a concentration of 1 x 10^6/mL in 10 mL of 50 mmol Tris-HCl, *bis*-Tris-HCl or *bis*-Tris-propane buffers (pH 7.0). Samples were placed into pouches and heat-sealed. Spore suspensions were heat-shocked at 80°C for 10 min and cooled in ice water prior to pressurization. The spore samples were pressurized at 4,000 atm at 25 or 45°C for 30 min. Samples of *B. subtilis* were serially diluted, pour-plated with NA and incubated at 37°C for 24 h. Samples of PA 3679 were serially diluted, pour-plated with RCM with 1.5% agar added and incubated anaerobically at 37°C for 48 h.

Sensitivity to sodium nitrite, sodium acetate and benzoic acid

Spores of *B. subtilis* and PA 3679 were subjected to various concentrations of sodium nitrite, sodium acetate and benzoic acid (sodium salt) to determine their sensitivity to these additives. This information would provide the level needed to give a 2-3 $\log_{10}$ reduction, so that possible synergy of these additives with pressure could be examined. Spore suspensions of *B. subtilis* and PA 3679 were diluted to a concentration of 1 x 10^6/mL in McIlvane's citrate-phosphate buffer (pH 7.0 or 4.0). Samples were placed into pouches serially diluted and pour-plated in NA amended with 0.1 to 4.0%

sodium nitrite, 0.1 to 5.0% benzoic acid and 0.1 to 5.0% sodium acetate. The plates were incubated at 37°C for up to 4 days. Spore suspensions of PA 3679 at a concentration of 1 x 10^6/mL were serially diluted and pour-plated in RCM amended with 0.02 to 5.0% sodium nitrite, 0.1 to 5.0% benzoic acid and 0.1 to 5.0% sodium acetate. The plates were incubated anaerobically at 37°C for up to 4 days.

Pressure combined with additives

Spore suspensions of *B. subtilis* and PA 3679 were diluted to a concentration of 1 x 10^6/mL in McIlvaine's citrate-phosphate buffer (pH 7.0 or 4.0). Samples were placed into pouches, heat-sealed, heat-shocked at 80°C for 10 min and chilled in ice water. The spore samples were pressurized at 4,000 atm at 25 or 45°C for 15 or 30 min. Samples pressurized at 45°C were cooled in ice water after treatment. Spores of *B. subtilis* were serially diluted and pour-plated in NA amended with 0.5 and 1.0% sodium nitrite or 0.5 and 1.0% sodium acetate before and after pressurization. Plates were incubated at 37°C for up to 48 h. Samples of PA 3679 were serially diluted and pour-plated in RCM amended with 0.06% sodium nitrite before and after pressurization. Plates were incubated anaerobically for 48 h.

Pressurization of spores at 5,100 atm (at Oregon State University)

Spores of *B. subtilis* 168 and *C. sporogenes* PA 3679 were placed into McIlvaine's citrate phosphate buffer (pH 4.0, 5.0, 6.0 and 7.0) at a concentration of 1 x 10^6 spores/mL. Samples were taken and plated to determine initial spore concentrations. Ten mL of spore suspension was placed into Kapak pouches (in duplicate pouches), sealed, heat-shocked at 80°C for 10 min and immediately chilled in ice water. The samples were shipped overnight to Oregon State University and kept refrigerated until pressure-treatment. The samples were heat-shocked immediately prior to pressurization. Samples were

pressurized at 5,100 atm (75,000 psi) at 25°C for 30 min in an Englewood Pressure System Inc, pilot plant pressurization unit . The samples were shipped back to Delaware overnight and stored at 4°C until sampled. Samples were heat-shocked and serially diluted. Spore suspensions of *B. subtilis* were pour-plated in NA and incubated at 37°C for 3 days. Spore suspensions of PA 3679 were pour-plated in RCM and incubated anaerobically at 37°C for 3 days.

Sensitivity to sucrose laurate

Spores of *B .subtilis* 168 and *C. sporogenes* PA 3679 were subjected to various concentrations of sucrose laurate (L-1695, Mitsubishi-Kasei Foods Corporation, Tokyo, Japan) to determine their sensitivity to this additive. These data provided the concentration of sucrose laurate necessary to give a 2 to 3-$\log_{10}$ reduction, so that possible synergy of pressurization and sucrose laurate could be determined. Spore suspensions of *B. subtilis* at a concentration of 1×10^6/mL were serially diluted and pour-plated in NA amended with 0.1 to 4.5% sucrose laurate. The plates were incubated at 37°C for up to 3 days. Spore suspensions of PA 3679 at a concentration of 1×10^6/mL were serially diluted and pour-plated in RCM amended with 0.05 to 2.0% sucrose laurate. The plates were incubated anaerobically at 37°C for up to 5 days. Sucrose laurate was also added to the pressure medium prior to pressurization.

Spore crops of mutant *Bacillus subtilis* strains

Six mutant strains of *B. subtilis* were obtained for the Bacillus Genetic Stock Center housed at Ohio State University. These strains, originally characterized by Peter Setlow, are lacking one or more of the small acid-soluble proteins (SASP) that have been shown to play a role protecting spore DNA from damage by heat. A spore crop for each strain of *Bacillus* was created by plating a 48-h vegetative

culture onto 100 NA plates and incubating at 37°C. Sporulation was assessed by phase-contrast microscopy. Spores were harvested after 6 days with sterile water and a bent-glass rod. Spore suspensions were stored at 4°C until purification procedure were performed.

Spore purification was done following the procedure as described by Nicholson and Setlow (1990). Spore crops were stored in sterile water at 4°C until needed.

Pressure combined with sucrose laurate

Spore suspensions of *B. subtilis* were diluted to a concentration of 1 x 10^6/mL in McIlvaine's citrate-phosphate buffer (pH 4.0, 5.0, 6.0 or 7.0). Control samples were taken. Samples were placed into pouches, heat-sealed, heat-shocked at 80°C for 10 min and chilled in ice water. The spore samples were pressurized at 4,000 atm at 25°C for 30 min or 45°C for 15 min. Samples pressurized at 45°C were cooled in ice water after treatment. Both control and pressure-treated spore samples were serially diluted and pour-plated in NA amended with 0.10. 0.25, 0.50, or 1.0% sucrose laurate. Plates were incubated at 37°C for up to 5 days.

A second method was also used to examine the combined effects of pressure and sucrose laurate on *B. subtilis* spores. Sucrose laurate was added to nutrient broth (NB) to concentrations of 0.1 to 1.0% prior to sterilization. Spores were added to a concentration of 1 x 10^6/mL and 10 mL was placed into pouches, heat-shocked, and pressurized as described above. Samples were serially diluted and pour-plated in NA. As there was a carry-over effect of the sucrose laurate observed when samples were pour-plated, a 1/10 dilution was done with 0.1%-peptone water prior to plating, therefore the lowest dilution able to be plated was 10^{-1}.

Inoculated pack studies

Reinforced clostridial medium (RCM) was brought to pH 6.5 or 6.0 with 1 M HCl prior to autoclaving. Spores of *Clostridium sporogenes* PA3679 were inoculated into RCM to concentrations of 10^2, 10^3 or 10^4 spores/mL. When used, sterilized nisin solutions were aseptically added to sterile RCM to concentrations of 0.05, 0.1, 1.0 or 5.0 IU/mL. Ten mL of spore suspension was aseptically transferred to Kapak pouches, heat-sealed, heat-shocked at 80°C for 10 min and immediately chilled in ice water. Pressure-treated samples were held in an ice-water bath until treated with combinations of 4,000 atm and 25 to 95°C for 15 or 30 min. Samples treated at temperatures above 25°C were immediately cooled in an ice-water bath. Pouches were incubated at 37°C and examined periodically for turbidity and gas production (swelling). Samples were taken prior to pressurization, pour-plated with RCM amended with 1.5% agar and anaerobically incubated at 37°C to determine initial spore concentrations. Two control pouches were taken for each trial. These pouches were heat- shocked only and incubated at 37°C. All control pouches showed turbidity and gas production within 3 d.

RESULTS AND DISCUSSION

Nisin effects on spore outgrowth

At a nisin concentration of 0.2 IU/mL, a 1-$\log_{10}$ reduction in CFU/mL was seen with the *B. subtilis* spores (Fig. 1). When the nisin level was increased to 1.0 IU/mL, a 3-$\log_{10}$ reduction was observed. A 2-$\log_{10}$ decrease in CFU/mL was seen when the nisin concentration was 0.6 IU/mL. The 0.6-IU/mL concentration was chosen for use in the high pressure protocol.

A nisin concentration of 1.0 IU/mL gave <1-$\log_{10}$ reduction in CFU/mL was observed for the PA 3679 spores (Fig. 2). By increasing the nisin level to 10.0 IU/mL, CFU/mL were decreased by 2 $\log_{10}$. A 3-$\log_{10}$ decrease was seen when nisin concentration was raised to 100 IU/mL. At 500 IU/mL, spore outgrowth was not completely inhibited with an average of 3 CFU/mL still detectable. The 10.0-IU/mL concentration was chosen for use in the high pressure experiments with PA 3679.

Treatments of 4,000 atm at 25°C resulted in <1.0-$\log_{10}$ reduction in CFU/mL for both types of spores, with the exception of a 2-$\log_{10}$ reduction when PA 3679 spores were treated in pH-4.0 buffer (Fig. 3 and 4). Treatments at 45°C gave 0.5- to 3.5-$\log_{10}$ reductions for *B. subtilis* spores treated for 15 or 30 min (Fig.5) and for PA3679 spores treated for 15 min (Fig. 6). Sterility was achieved when the PA 3679 spores were treated in pH 4.0 buffer for 30 min at 45°C. Spore suspensions of *B. subtilis* showed a 6-$\log_{10}$ reduction in CFU/mL when pressurized at 70°C and pH 5.0, 6.0 and 7.0. Sterility was achieved with pressurization for 15 min at pH 4.0 and 70°C (data not shown). Spore suspensions of PA 3679 treated at 70°C and pH 4.0 or 5.0 showed 4- to 6-$\log_{10}$ reductions, but only 1.0-$\log_{10}$ reduction at pH 6.0 or 7.0 (Fig. 7). By increasing the temperature to 90°, the PA 3679 spore suspensions were sterilized at pH 4.0, 5.0, and 6.0 (data not shown).

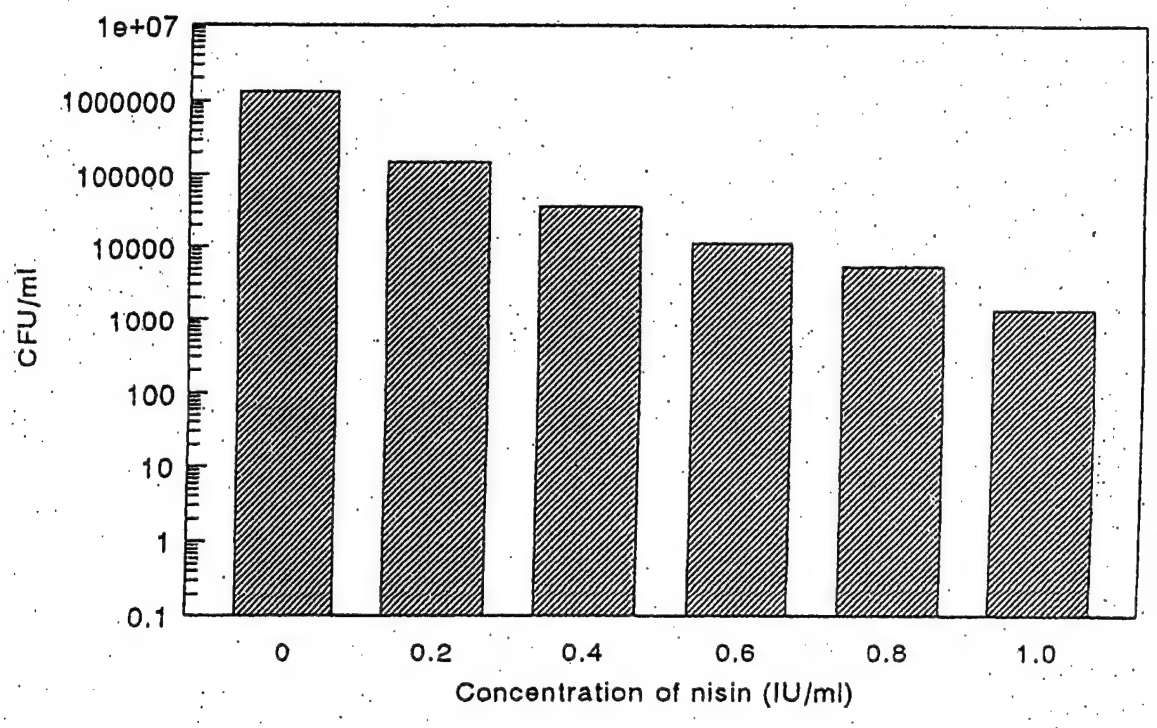


Figure 1. Effect of nisin on the growth of *B. subtilis* spores when cultured on nutrient agar amended with nisin

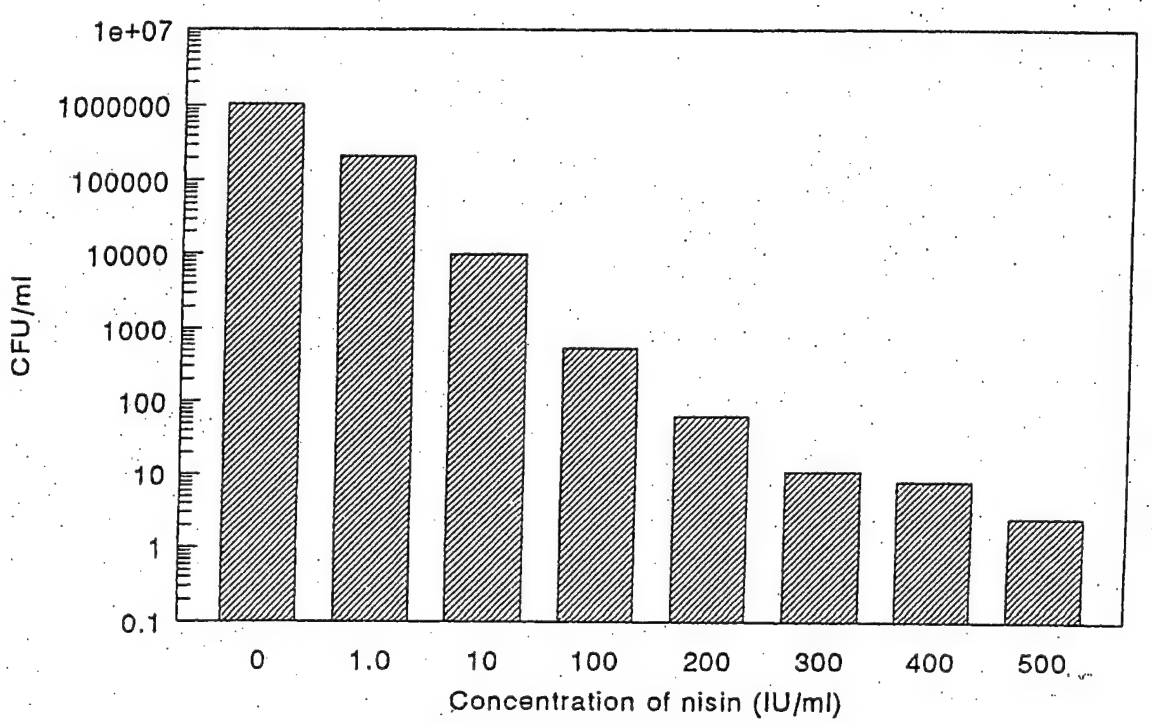


Figure 2. Effect of nisin on the growth of PA3679 spores when cultured on nutrient agar amended with nisin

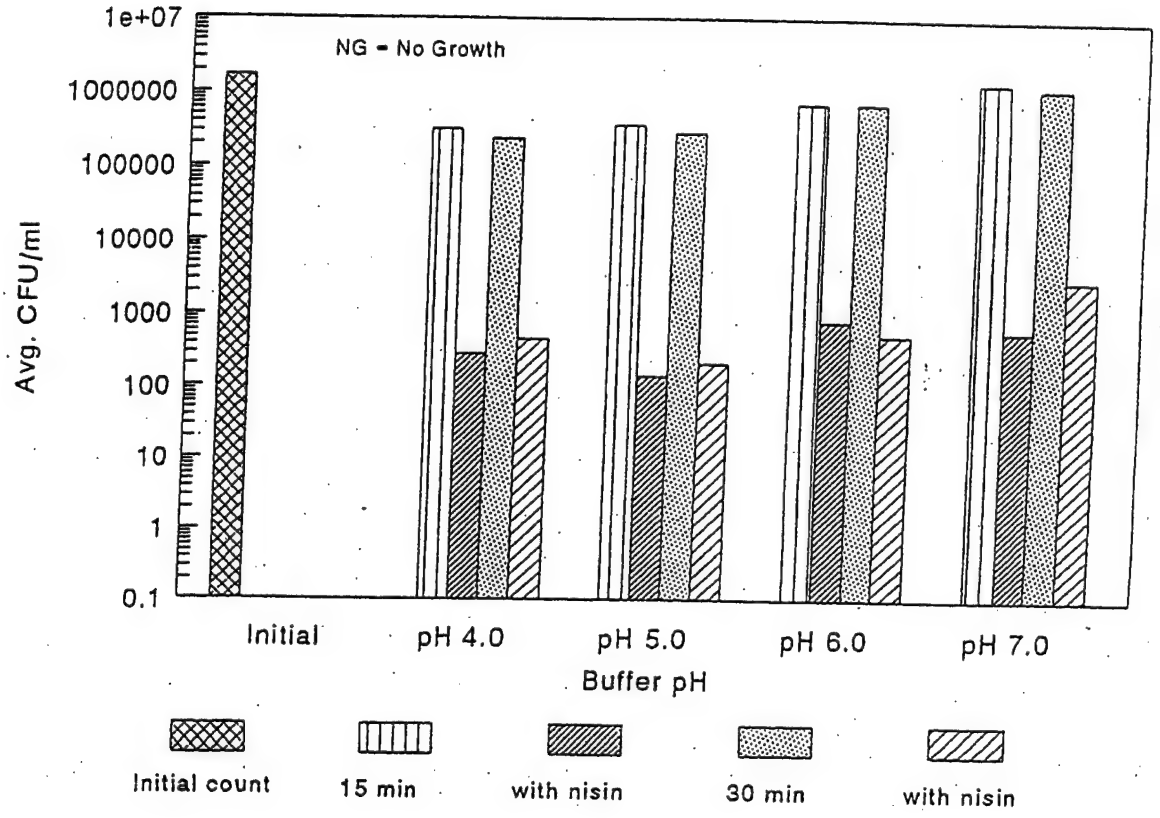


Figure 3. Effect of high hydrostatic pressure (4000 atm.) on the growth of *B. subtilis* spores incubated (25°C) in different buffers (pH 4 – 7) containing 0.6 IU/mL of nisin

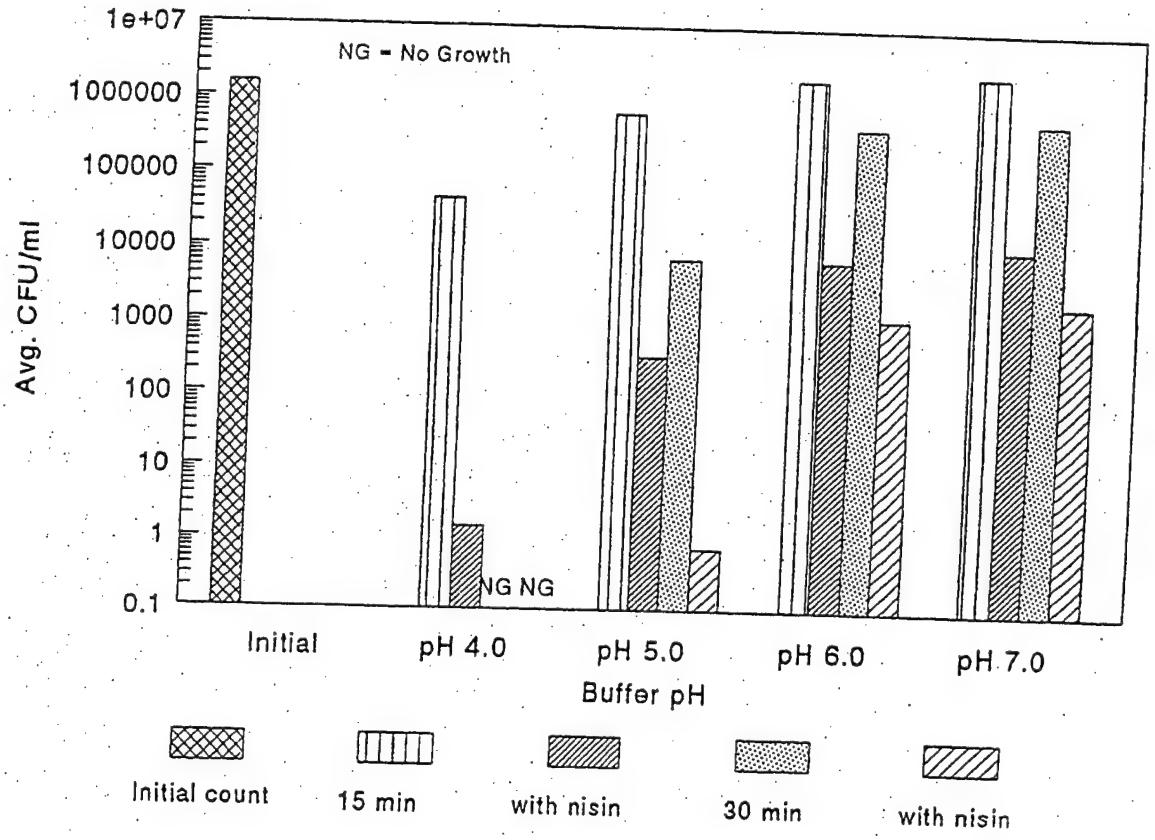


Figure 4. Effect of high hydrostatic pressure (4000 atm.) on the growth of PA3679 spores incubated (25°C) in different buffers (pH 4 – 7) containing 10 IU/mL of nisin

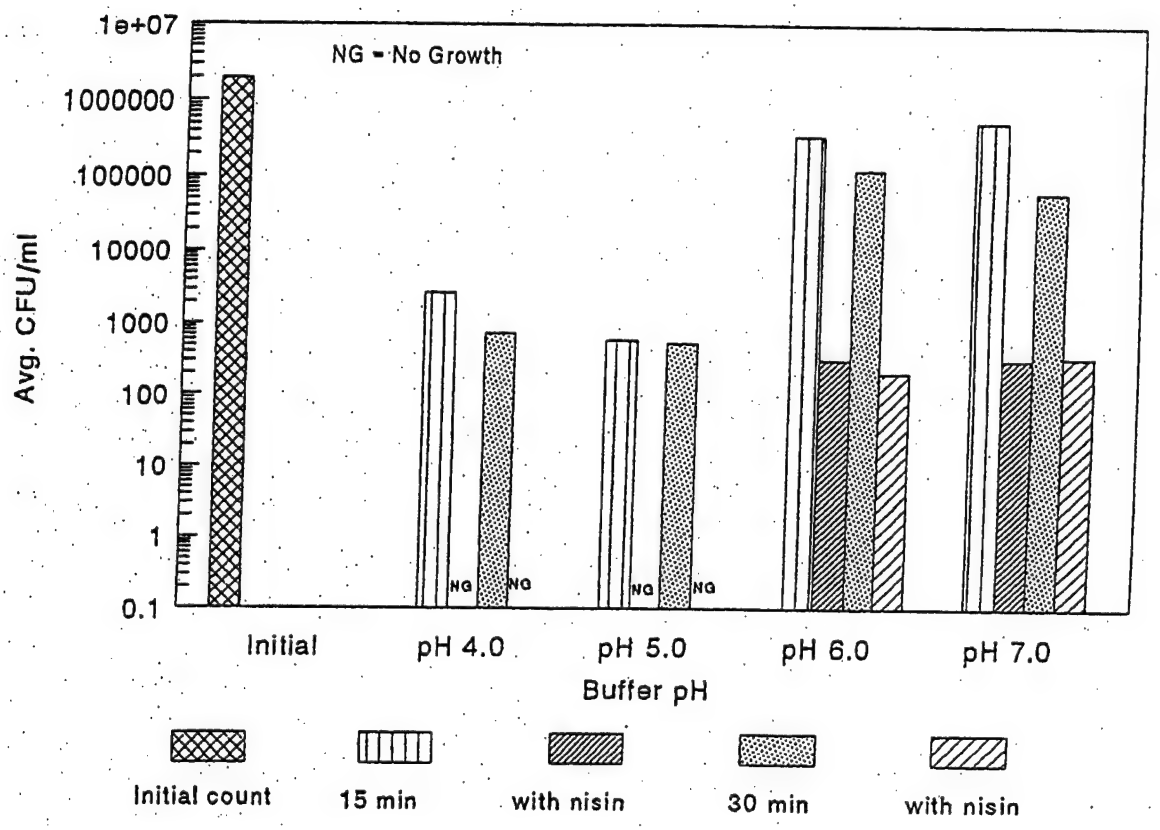


Figure 5. Effect of high hydrostatic pressure (4000 atm.) on the growth of *B. subtilis* spores incubated (45°C) in different buffers (pH 4 – 7) containing 0.6 IU/mL of nisin

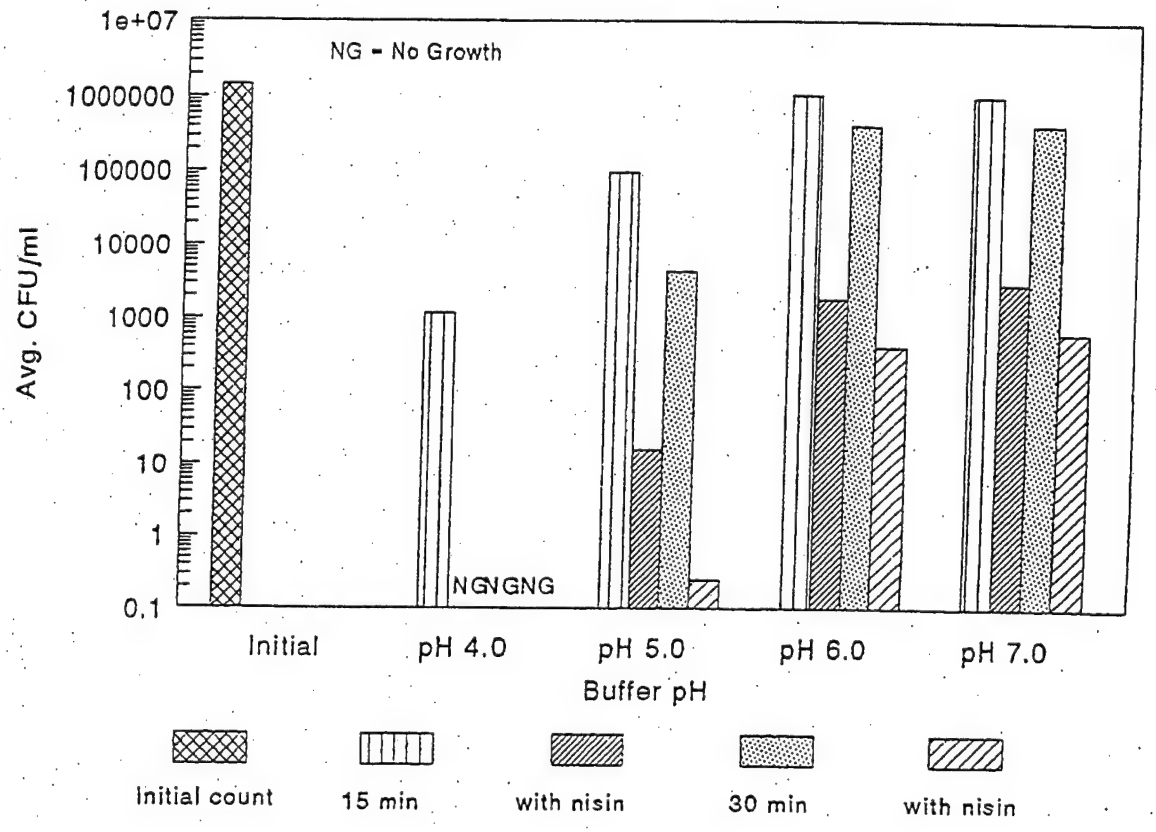


Figure 6. Effect of high hydrostatic pressure (4000 atm.) on the growth of PA3679 spores incubated (45°C) in different buffers (pH 4 – 7) containing 10 IU/mL of nisin

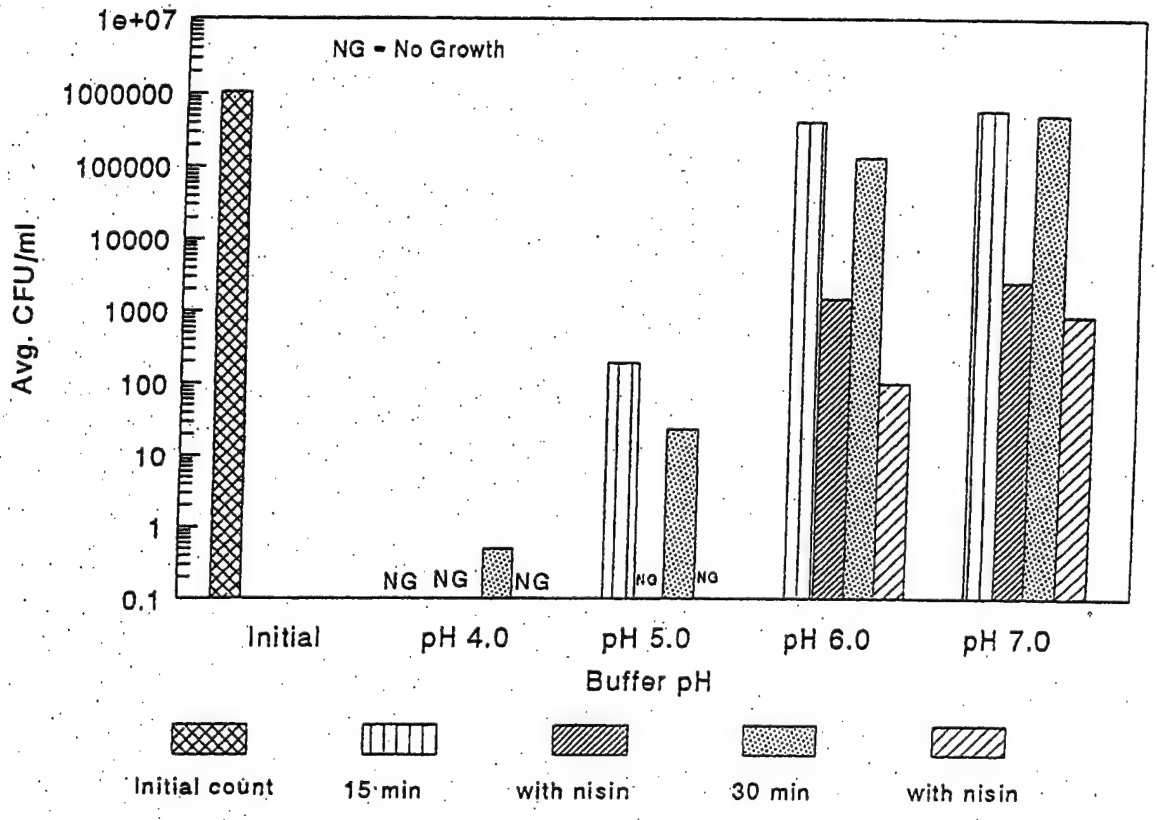


Figure 7. Effect of high hydrostatic pressure (4000 atm.) on the growth of PA3679 spores incubated (70°C) in different buffers (pH 4 – 7) containing 10 IU/mL of nisin

When pressure-treated spores were plated in media containing nisin, additional 3- to 4-$\log_{10}$ reductions were observed with the PA 3679 samples. Additional 3-$\log_{10}$ reductions were observed with the samples of *B. subtilis*. This suggests that nisin, when added to the plating medium, possibly has a synergistic effect with the other parameters (pH and pressurization) tested.

Pressure oscillations

A 1-$\log_{10}$ cycle or less reduction in CFU/mL was observed for the majority of *B. subtilis* samples pressurized at 25°C regardless of the pH of the buffer or the number of oscillations (Fig. 8). The greatest reduction was seen with eight oscillations at pH 4.0 with a 1.5-$\log_{10}$ cycle decrease in spore counts. These results are consistent with the data on pressurization of *B. subtilis* spores without oscillation for 15- and 30-min constant pressure exposure times. When the temperature during pressurization was raised to 45°C, the number of *B. subtilis* spores capable of outgrowth was dramatically decreased (Fig. 9). A 3-cycle oscillation treatment at pH 4.0 resulted in complete inactivation of 10^6 spores/mL, as compared to a 3-$\log_{10}$ cycle decrease when pressurized for 15 min without oscillation (Fig. 9a). The 4- and 6-cycle oscillations of pressure at 45°C gave a 6-$\log_{10}$ cycle reduction in CFU/mL for samples pressurized at pH 4.0, 5.0 and 6.0. Those samples pressurized at pH 7.0 were reduced approximately 3- and 4-$\log_{10}$ cycles, respectively. The implementation of pressure oscillation gave a greater decrease in surviving spore counts as compared to 30-min pressure treatments without oscillation. Without oscillation, spore samples were reduced approximately 3.5-$\log_{10}$ cycles when pressurized at 4,000 atm at 45°C for 30 min, pH 4.0 or 5.0. At pH 6.0 the reduction was about 1 $\log_{10}$ and at pH 7.0 the reduction was about 1.5 $\log_{10}$ cycles.

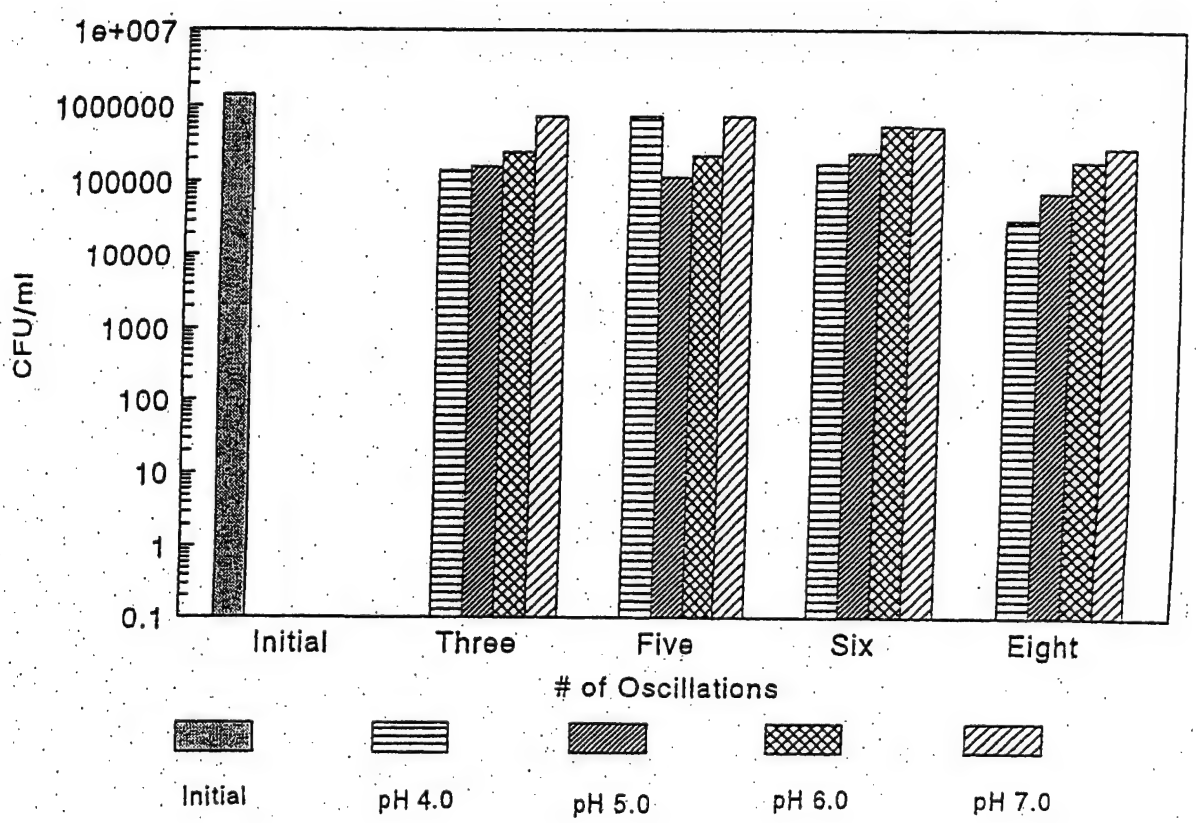


Figure 8. Effect of 5 minute multiple pressure (4000 atm.) oscillations (pH 4 – 7) at 25°C on the outgrowth of spores of *B. subtilis* 168

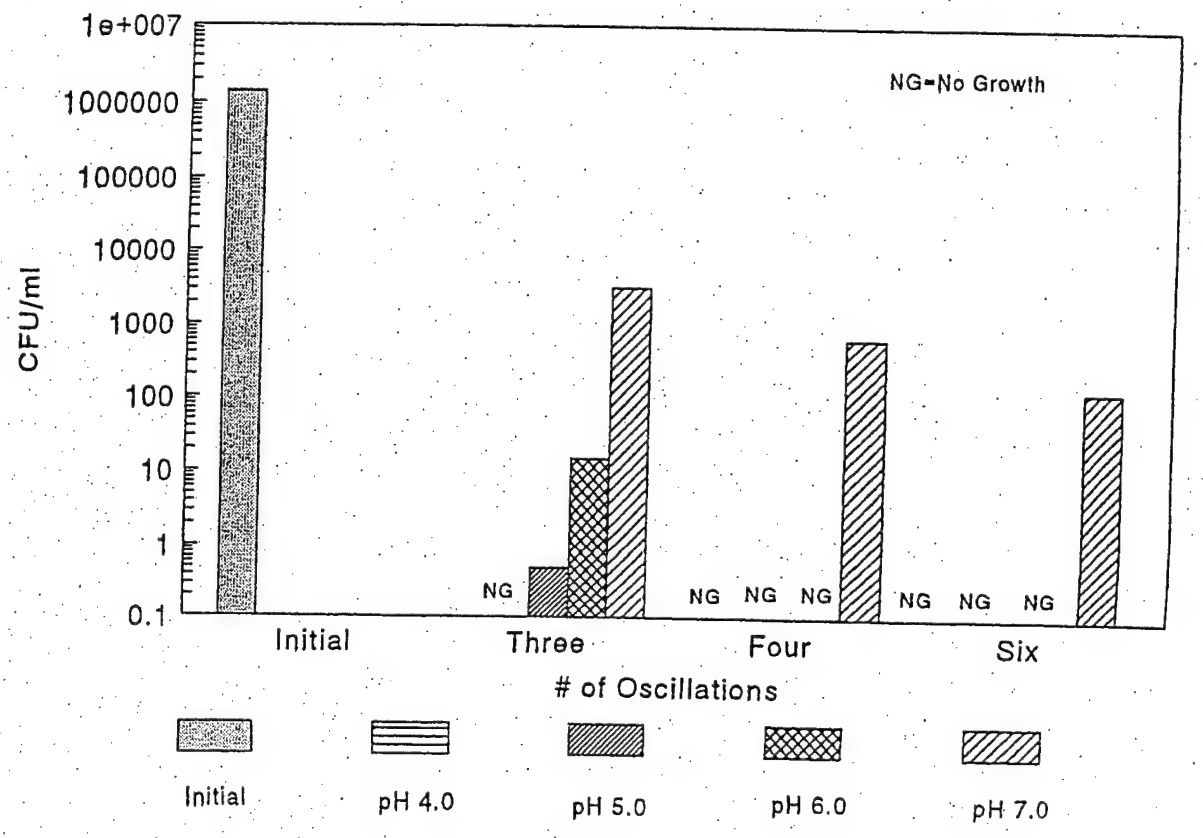


Figure 9. Effect of 5 minute multiple pressure (4000 atm.) oscillations (pH 4 – 7) at 45°C on the outgrowth of spores of *B. subtilis* 168

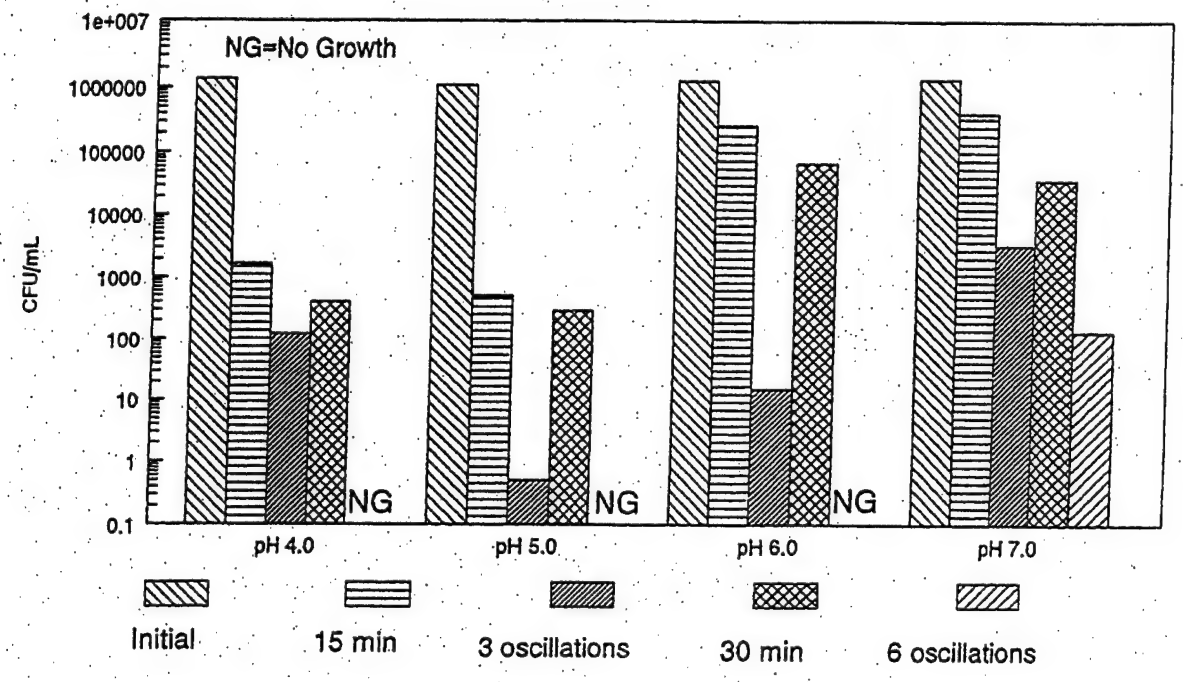


Figure 9a. Effect of 5 minute 3- and 6-cycle pressure (4000 atm.) oscillations (pH 4 – 7) at 45°C on the outgrowth of spores of *B. subtilis* 168

Buffer composition effects

There was no significant difference in plate counts seen among acetate, citrate and citrate-phosphate buffers used when *B. subtilis* spores were pressurized at 25°C and 4,000 atm for 30 min at pH 5.0 (Fig. 10). When pressurized at 45°C, the acetate and citrate-phosphate buffers gave similar results. The samples treated in citrate buffer were reduced an additional 1-$\log_{10}$ cycle (Fig. 11). The spore suspensions of PA 3679 pressurized in citrate-phosphate buffer have at either 25 or 45°C (McIlvaine buffer normally used in the pressurization studies) gave a 1- to 1.5-$\log_{10}$ cycle greater reduction in CFU/mL as compared to those pressurized in acetate or citrate buffers.

Spore suspensions of 1 x 10^6/mL *B. subtilis* were prepared and treated as described above but not subjected to pressure to determine the effect of elevated temperature (90°C) on spore outgrowth (Fig. 12). An exposure of 15 min at pH 5.0, 6.0, or 7.0 gave less than 0.5-$\log_{10}$ CFU/mL reductions. At pH 4.0, reductions of approximately 2-$\log_{10}$ cycles were observed. An exposure of 30 min at pH 6.0 or 7.0 showed no significant decrease in CFU/mL, pH 5.0 showed approximately a 2-$\log_{10}$ cycle reduction in CFU/mL, and no spores able to outgrow when heated to 90°C for 30 min at pH 4.0.

The Tris-HCl, bis-Tris-HCl and bis-Tris-propane buffers were compared along with the results obtained for pressurization of spore samples in McIlvaine's citrate-phosphate buffer. There were no differences seen among buffers with pressurization of either *B. subtilis* or PA 3679 spore samples at either 25 or 45°C among any of the four buffers examined (Figures 13 and 14). The spore suspensions of *B. subtilis* showed no decrease in CFU/mL with pressurization for 30 min at 25°C in any of the four pH 7.0 buffers tested. Pressurization at 45°C resulted in approximately a 1.5-$\log_{10}$ reduction in CFU/mL for all buffers tested.

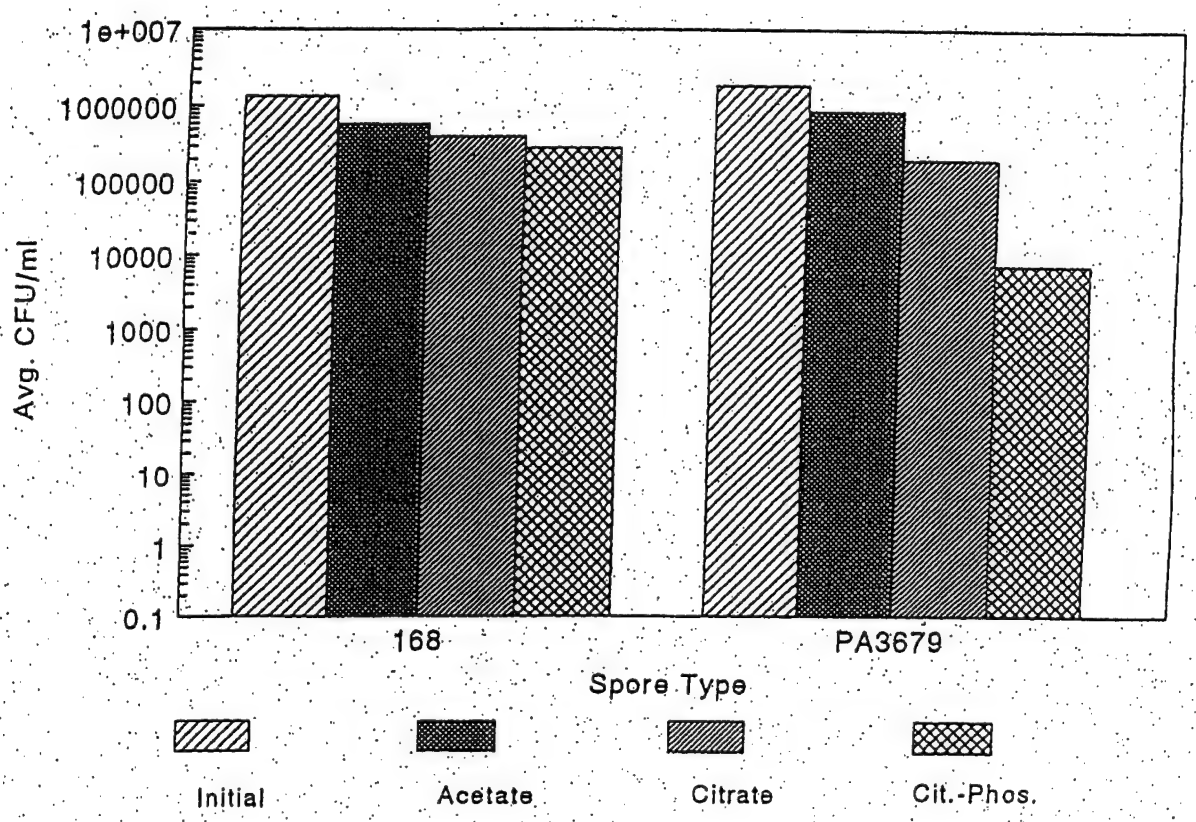


Figure 10. Effect of buffer type (pH 5.0) and pressure (4000 atm.) on the outgrowth of spores of *B. subtilis* 168 and PA3679 treated at 25°C.

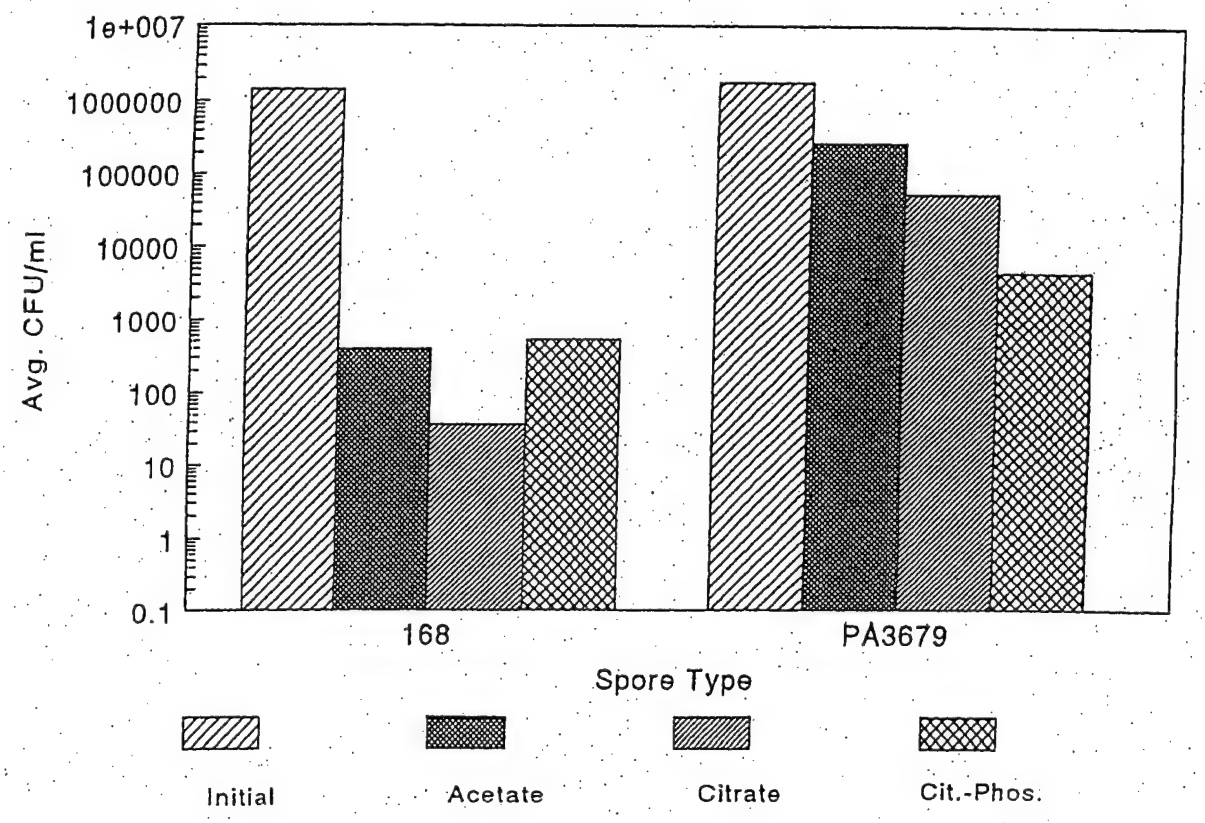


Figure 11. Effect of buffer type (pH 5.0) and pressure (4000 atm.) on the outgrowth of spores of *B. subtilis* 168 and PA3679 treated at 45°C

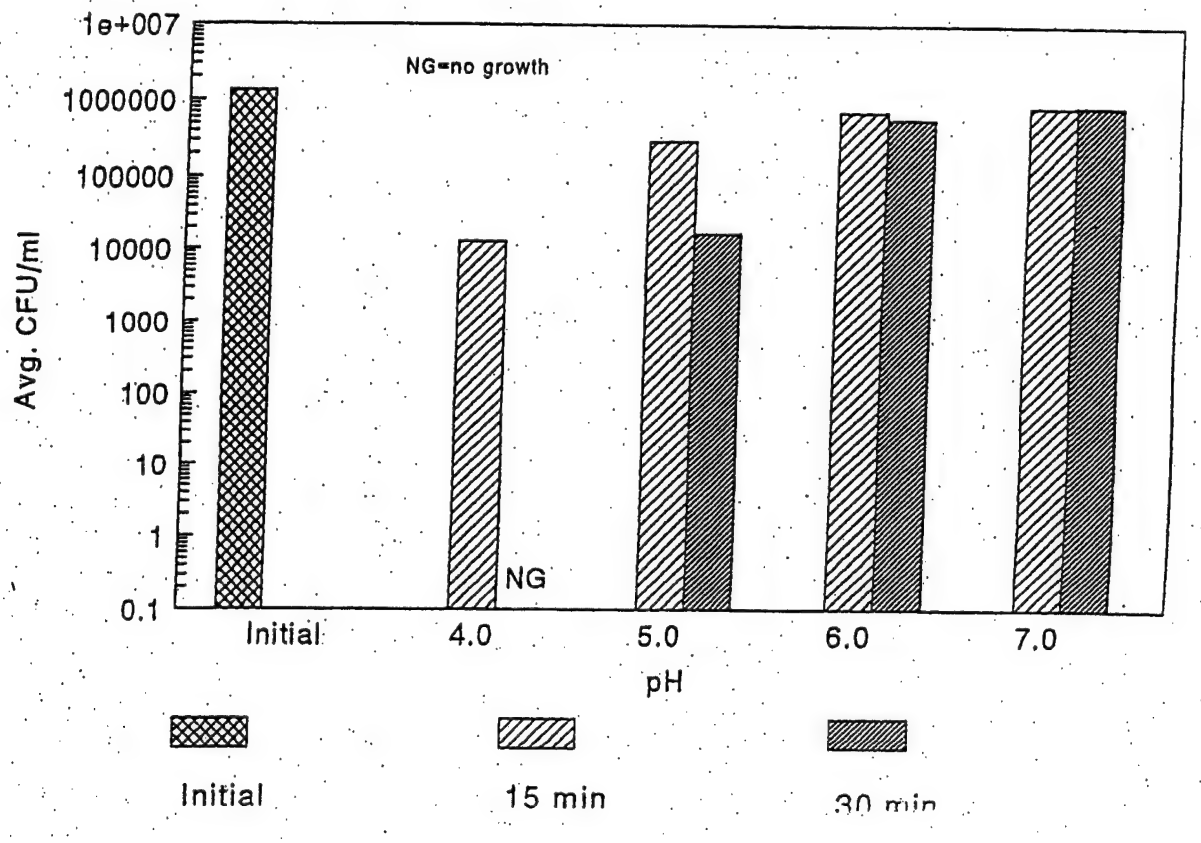


Figure 12. Effect of high temperature (90°C), pH (4.0-7.0) and time of exposure (15 or 30 min.) in the absence of pressure on the survival of *B. subtilis* 168

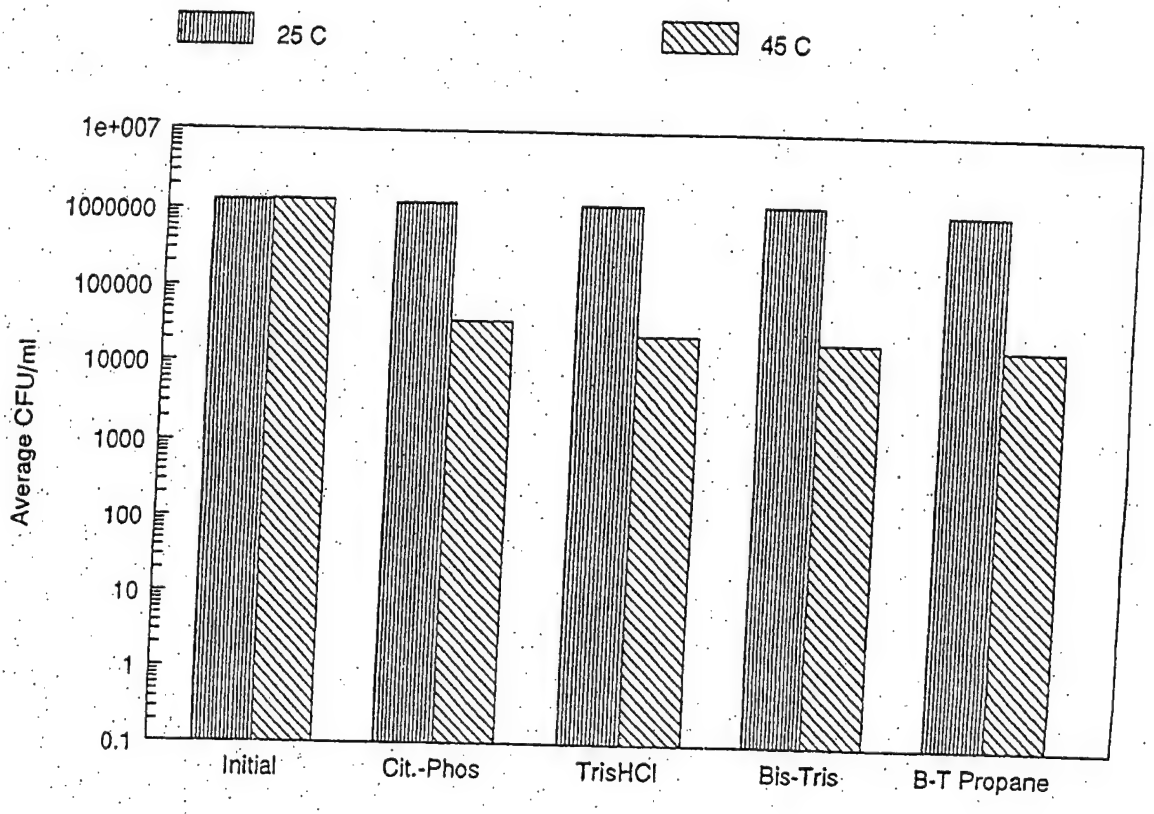


Figure 13. Survival of *B. subtilis* 168 treated in different pressurized (4000 atm) buffers (pH 7.0) at 25° and 45°C for 30 min.

Pressurization of the spore suspensions of PA 3679 showed no decrease in CFU/mL for any buffer used at either temperature (Fig. 14)

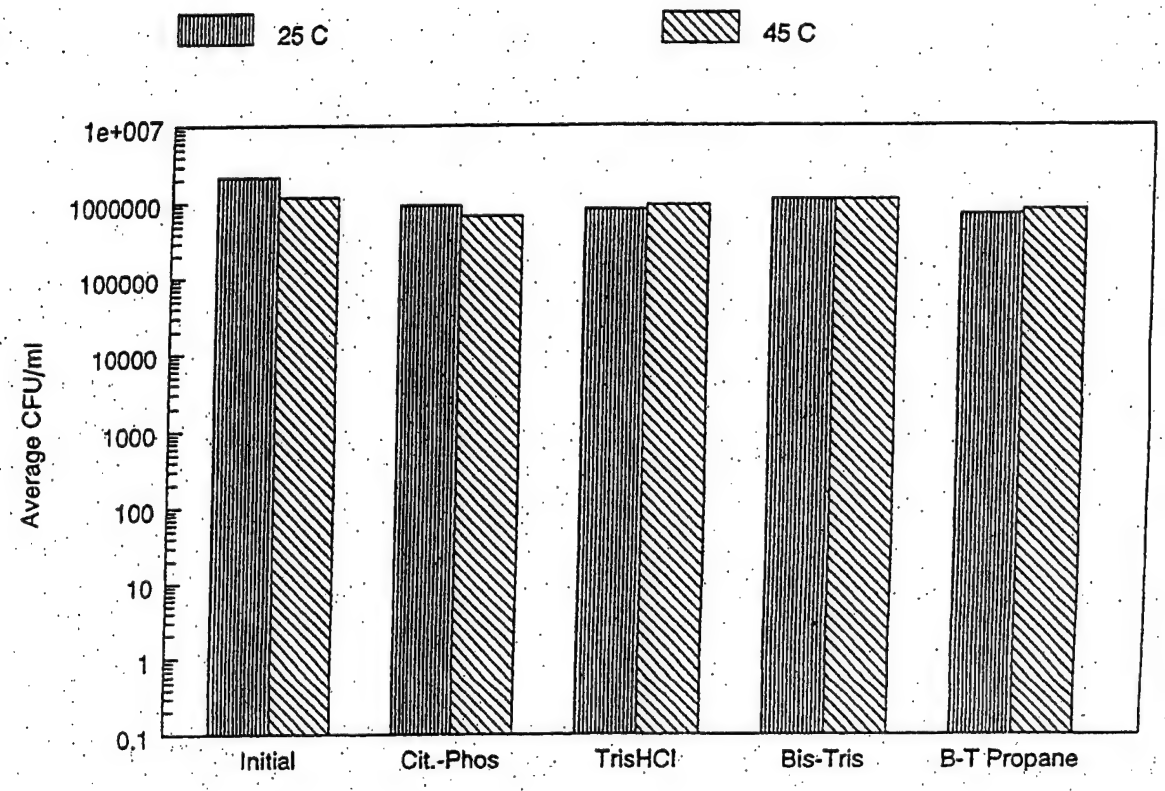


Figure 14. Survival of PA3679 treated in different pressurized (4000 atm) buffers (pH 7.0) at 25° and 45°C for 30 min

Food additive effects on spores

Spore suspensions of *B. subtilis* showed no decrease in CFU/mL when plated in NA containing up to 5.0% sodium nitrite or 5.0% sodium acetate (data not shown). Benzoic acid gave no decrease in CFU/mL at concentrations of 0.1 and 0.5%. Approximately 0.5-$\log_{10}$ reduction in CFU/mL was seen at the 0.7% level and no growth was observed after 3 days of incubation, when the benzoic acid concentration was raised to 1.0, 2.0 and 5.0%.

Spore suspensions of PA 3679 responded to the additives differently than *B. subtilis* 168 spores (data not shown). Sodium nitrite totally inhibited spore outgrowth at concentrations of 0.5% and higher. A 0.1% concentration gave a 2-$\log_{10}$ reduction in CFU/mL. Concentrations of nitrite from 0.04 to 0.08% gave approximately a 1.0-$\log_{10}$ reduction. The 0.02% concentration reduced CFU/mL by approximately 0.5-$\log_{10}$. Sodium acetate did not inhibit spore outgrowth at concentrations ranging from 0.1 to 5.0%. Benzoic acid gave no reduction in CFU/mL at the 0.1, 0.5 or 1.0% levels. Raising the concentration to 2.0% gave mixed results. The colonies that grew on the 10^{-1}-dilution plates were extremely small and too numerous to count. The 10^{-2}-dilution plates displayed no colony growth. A 5.0% concentration of benzoic acid fully inhibited outgrowth.

No apparent synergistic effect was observed when spore suspensions of *B. subtilis* were pressurized at 4,000 atm at 25°C for 30 min in pH 4.0 or 7.0 buffers, and subsequently plated on NA amended with 0.5 or 1.0% sodium nitrite (< 0.5 $\log_{10}$ reductions; Figure 15). When the temperature during pressurization was increased to 45°C, a slight synergistic effect was suggested (Figure 16).

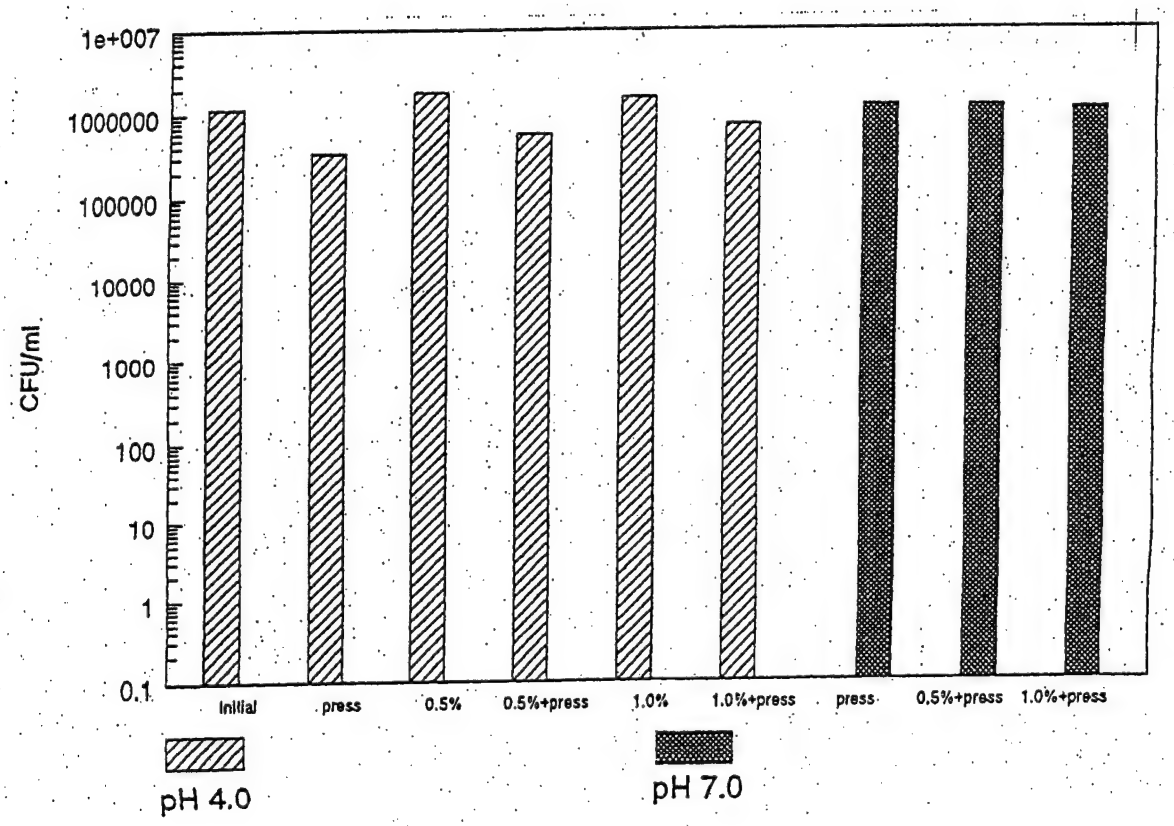


Figure 15. Survival of *B. subtilis* 168 spores pressurized (4000 atm) in buffer (pH 4.0 and 7.0) for 30 min at 25°C and plated on nutrient agar amended with 0.5% or 1.0% sodium nitrite

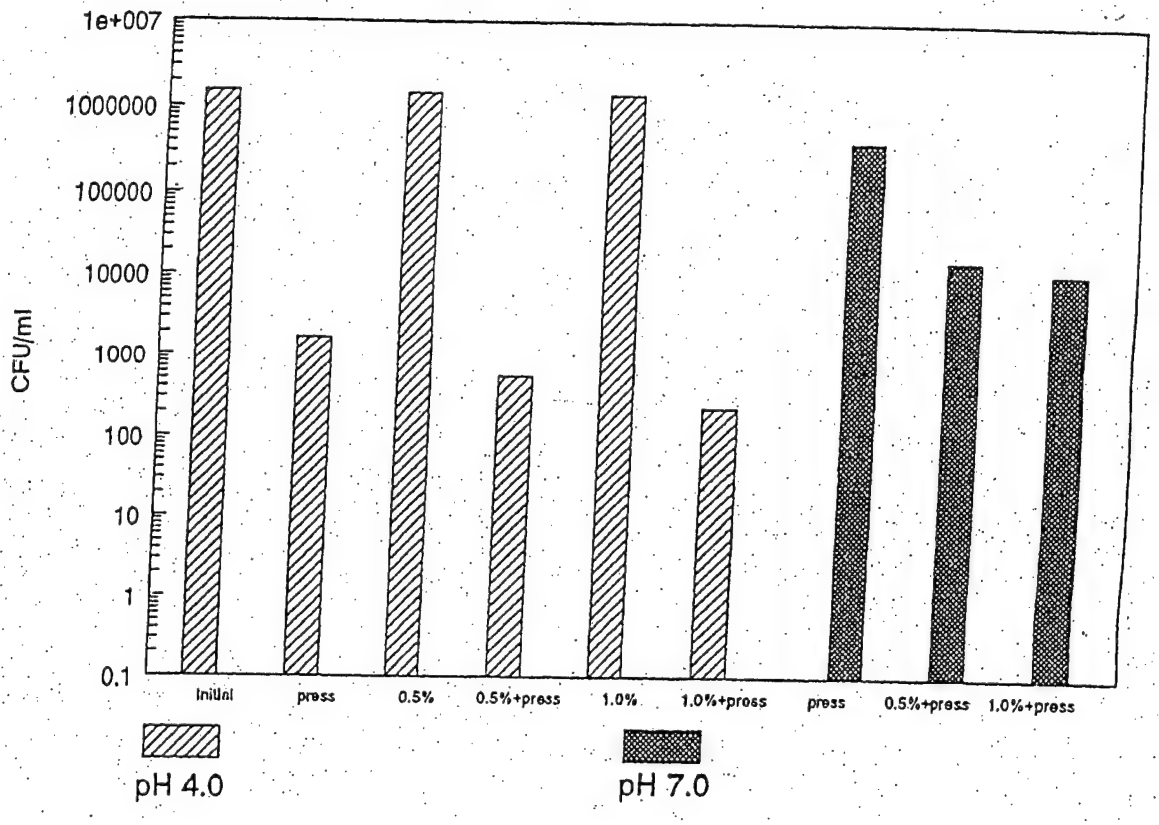


Figure 16. Survival of *B. subtilis* 168 spores pressurized (4000 atm) in buffer (pH 4.0 and 7.0) for 15 min at 45°C and plated on nutrient agar amended with 0.5% or 1.0% sodium nitrite

Pressurization at pH 4.0 gave an additional reduction of 0.5 and 1.0-$\log_{10}$ in CFU/mL when plated on NA containing 0.5 and 1.0% sodium nitrite, respectively. 1.0% nitrite. Pressurization in pH 7.0 buffer gave an additional 1.5-$\log_{10}$ reduction in CFU/mL when plated in NA containing either 0.5 or 1.0% nitrite. No synergistic effect was seen when spore suspensions of *B. subtilis* were pressurized at 4,000 atm and 25°C for 30 min or 45°C for 15 min in either pH 4.0 or 7.0 buffer followed by plating in NA containing 0.5 or 1.0% sodium acetate (Figures 17 and 18).

Spores suspensions of PA 3679 were subjected to 4,000 atm for 30 min in pH 7.0 buffer at 25 and 45°C. There was a minimal effect of pressure on these spores, but there was no synergistic effect observed for any treatments when the samples were plated on RCM amended with 0.06% sodium nitrite (Figure 19).

Pressurization of *B. subtilis* 168 spores (initial concentration: 1 x 10^6/mL) at the higher pressure of 5,100 atm, at 25°C for 30 min at pH 6.0 or 7.0 gave >3-$\log_{10}$ reduction in CFU/mL (Figure 20). Pressurization at pH 5.0 gave a 5-$\log_{10}$ decrease, and spore suspensions pressurized at pH 4.0 were reduced by 6-$\log_{10}$. The PA 3679 spore suspensions were more resistant than *B. subtilis* 168 to pressurization at 5,100 atm at pH 6.0 or 7.0 demonstrating only a 1.5-$\log_{10}$ reduction in CFU/ mL (Figure 21). Approximately a 3-$\log_{10}$ reduction was seen at pH 5.0 but a 6-$\log_{10}$ reduction was found with pressurization at pH 4.0.

Spores of *B. subtilis* 168 were found to be resistant to sucrose laurate. When spore suspensions with initial concentrations of 1 x 10^6/mL were plated in NA amended with 1.0 to 2.5% sucrose laurate, <1-$\log_{10}$ reductions in CFU/mL were observed (data not shown). Sucrose laurate concentrationson plating agar ranging from 3.0 to 4.5% all gave reductions of approximately 1.5-$\log_{10}$.

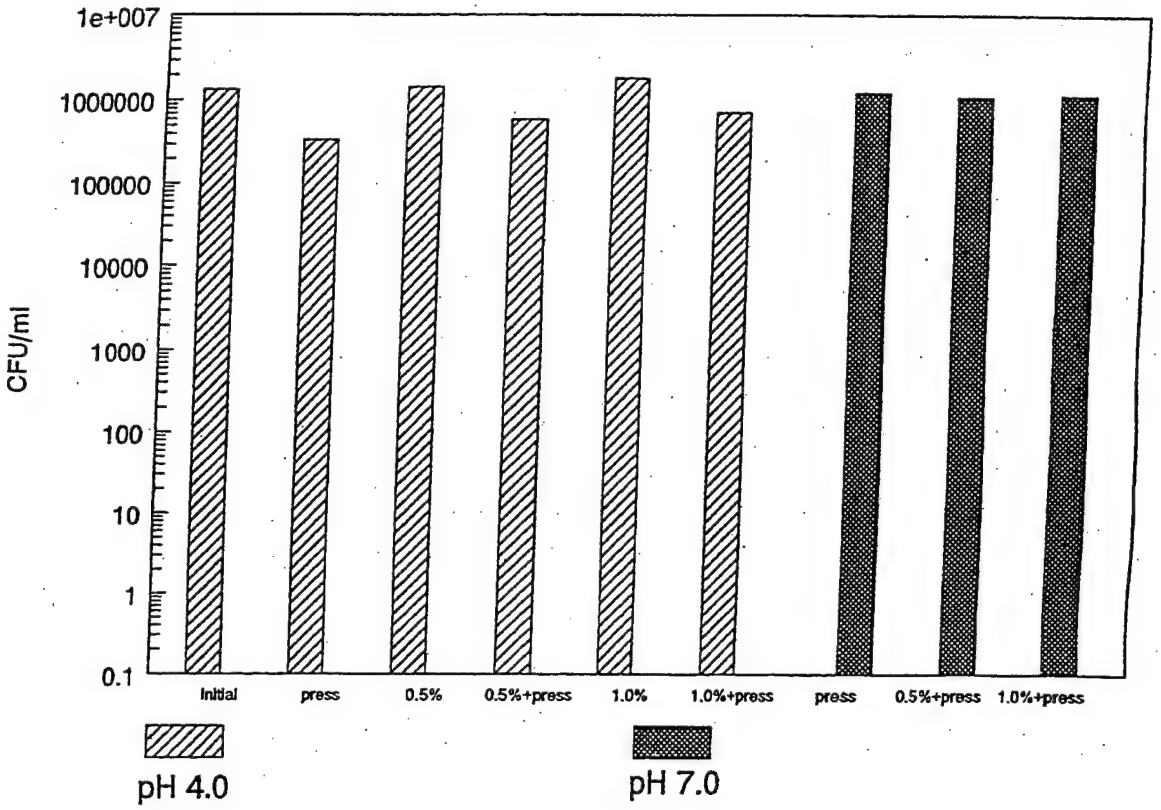


Figure 17. Survival of *B. subtilis* 168 spores pressurized (4000 atm) in buffer (pH 4.0 and 7.0) for 30 min at 25°C and plated on nutrient agar amended with 0.5% or 1.0% sodium acetate

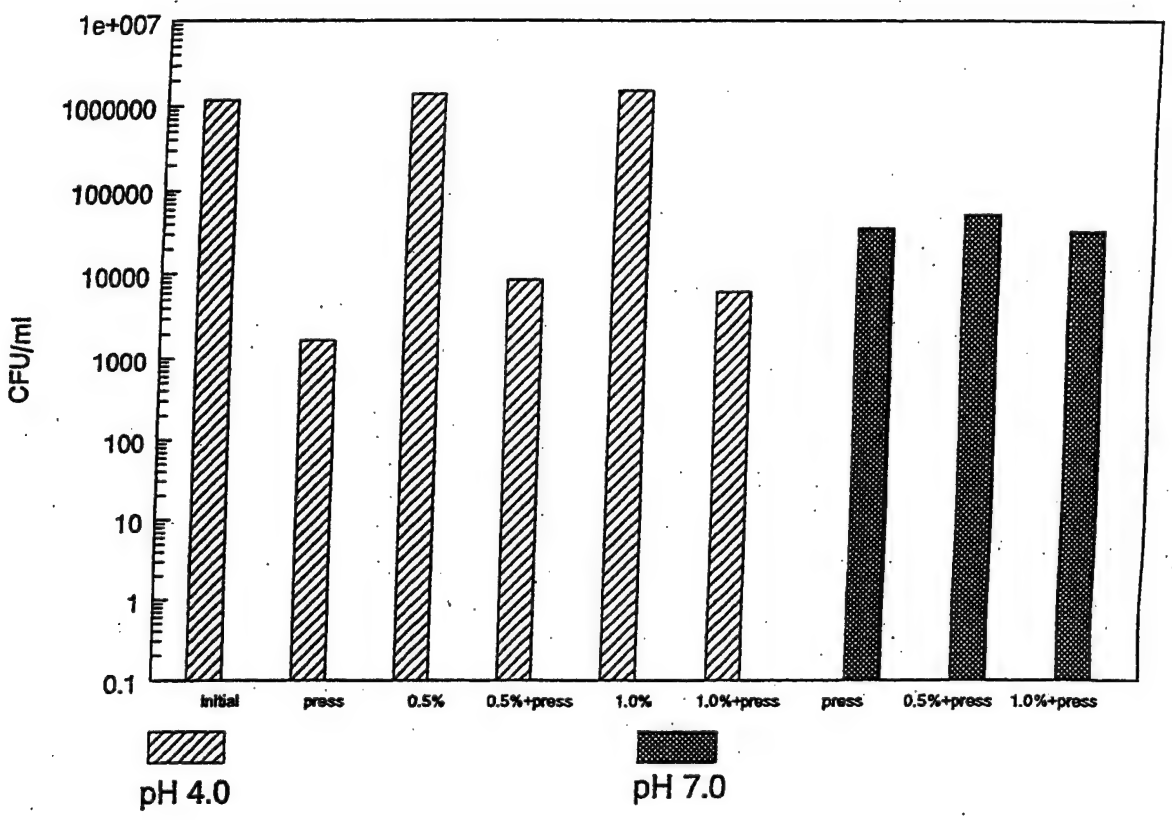


Figure 18. Survival of *B. subtilis* 168 spores pressurized (4000 atm) in buffer (pH 4.0 and 7.0) for 15 min at 45°C and plated on nutrient agar amended with 0.5% or 1.0% sodium acetate

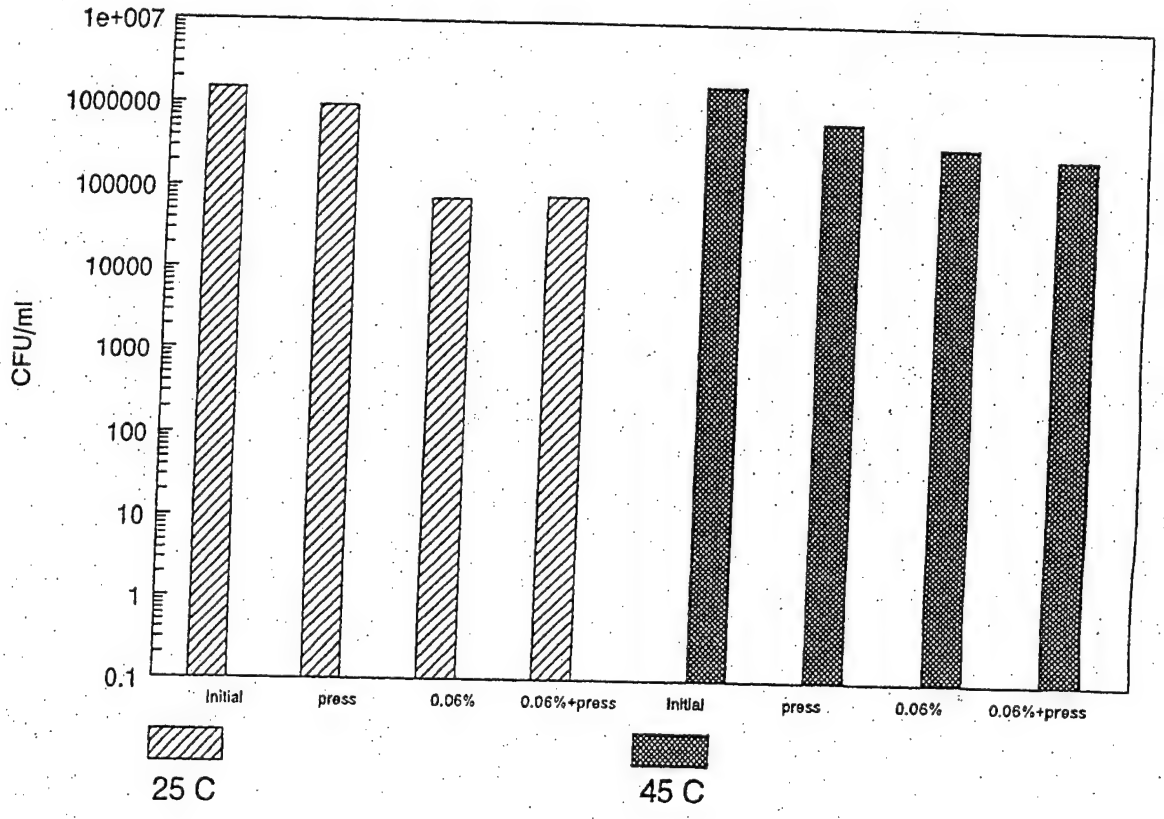


Figure 19. Survival of PA3679 spores pressurized (4000 atm) in buffer (pH 7.0) for 30 min at 25°C and 45°C and plated on RCM amended with 0.06% sodium nitrite

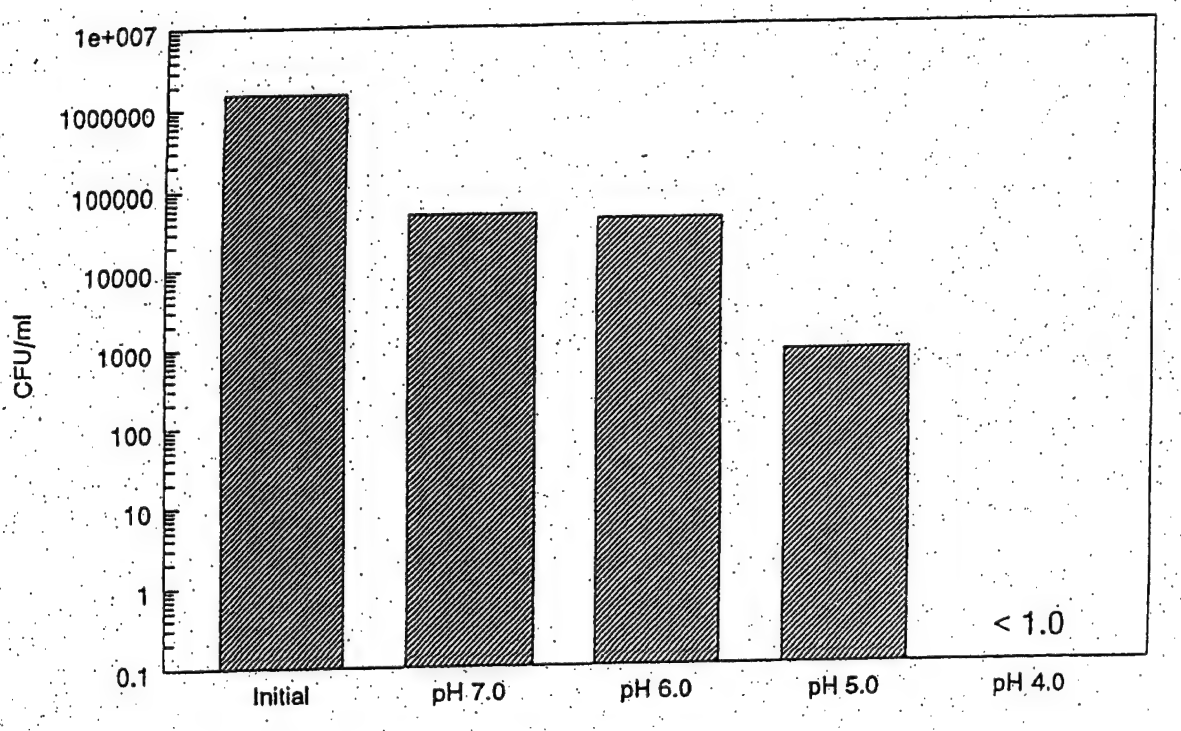


Figure 20. Effect of pressurization (5100 atm) on the survival of *B. subtilis* 168 spores treated at pH 4.0-7.0 for 30 min at 25°C

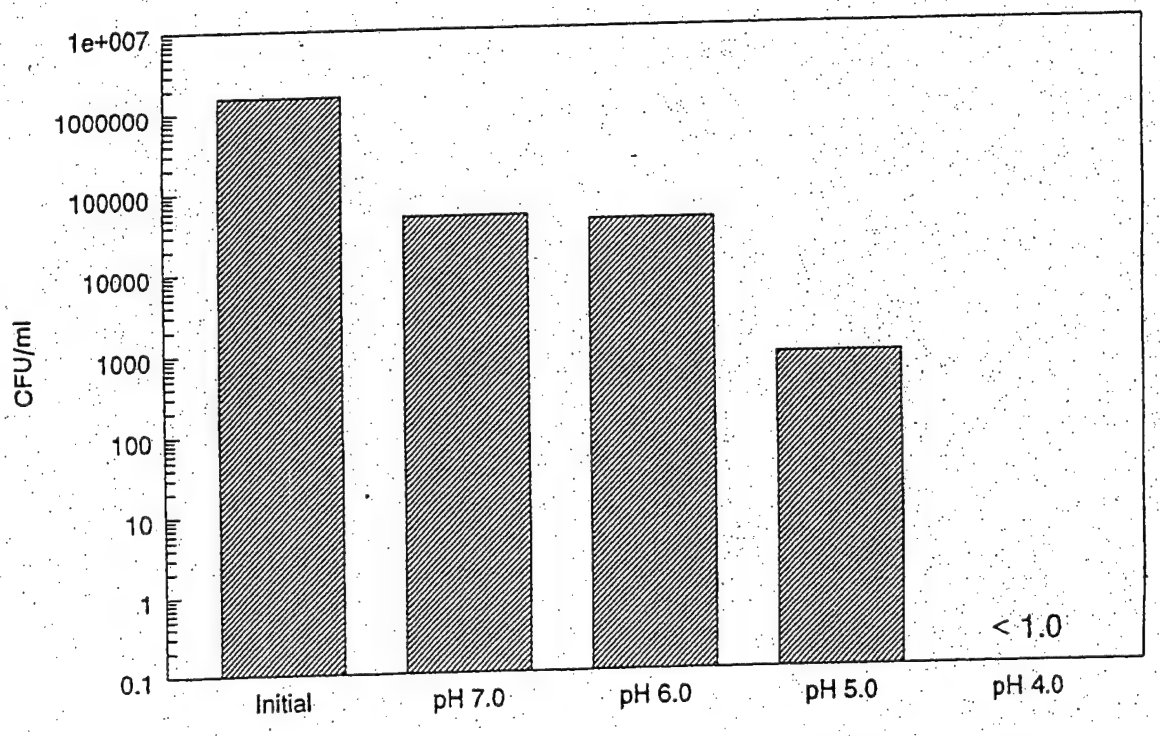
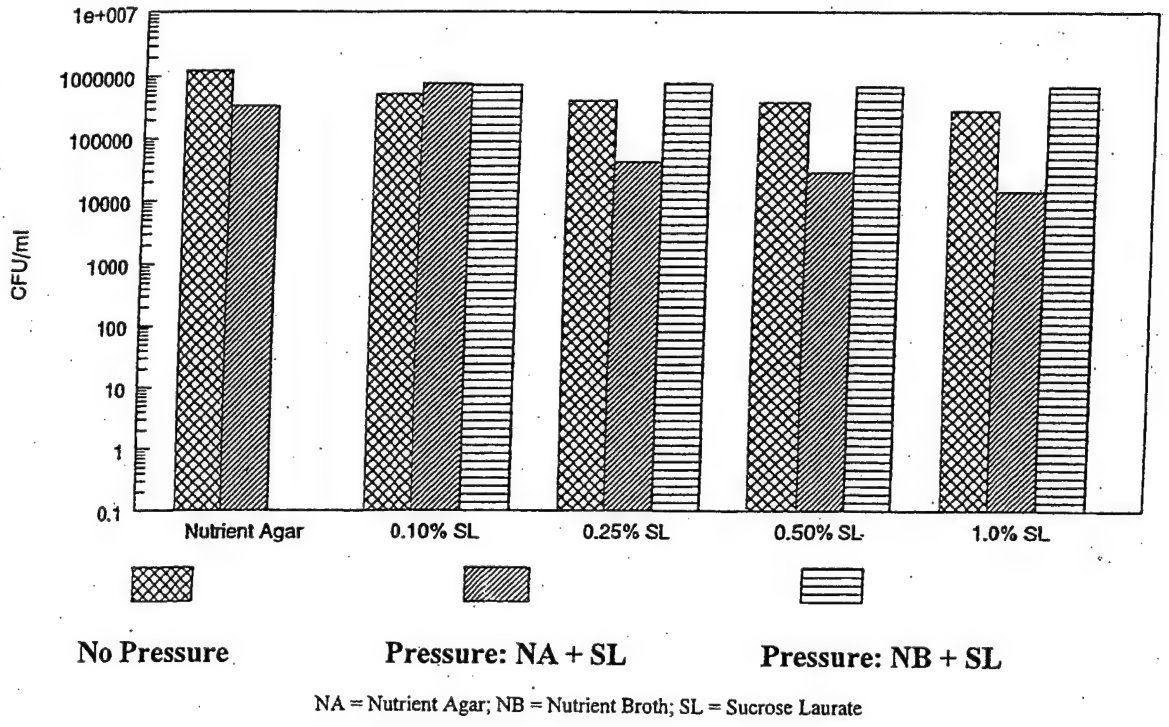


Figure 21. Effect of pressurization (5100 atm) on the survival of PA3679 spores treated at pH 4.0-7.0 for 30 min at 25°C

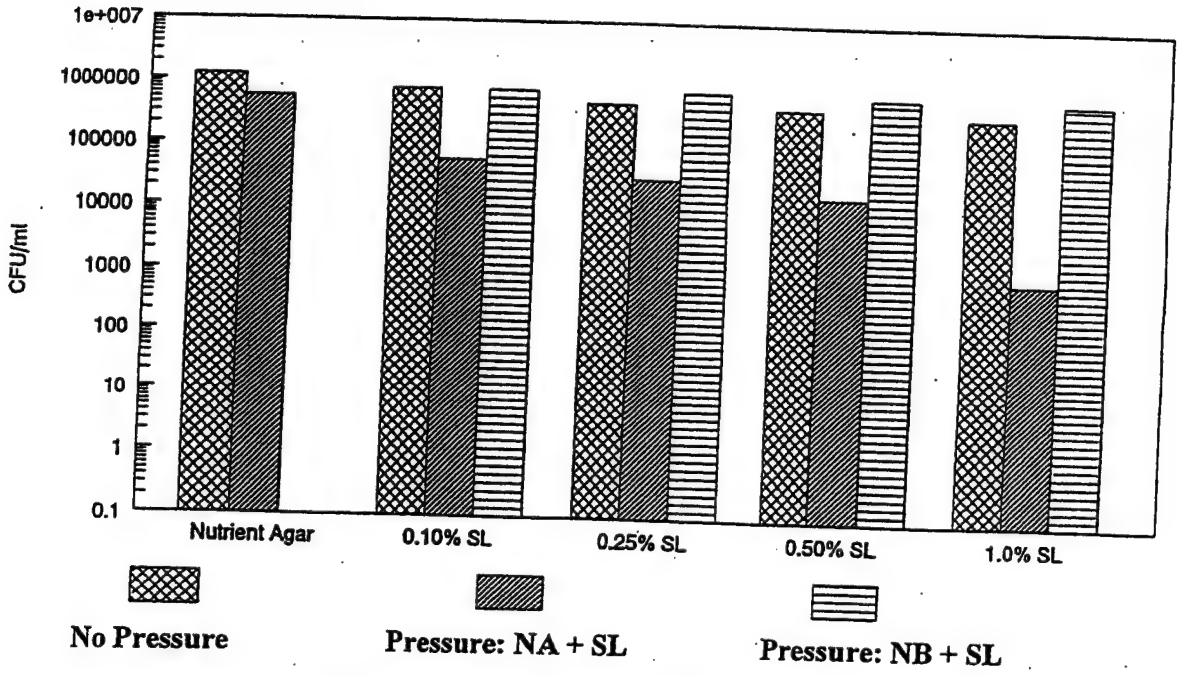
Spores of PA 3679 spores were extremely sensitive to sucrose laurate. When spore samples with an initial concentration of 1 x 10^6/mL were plated in RCM amended with as little as 0.05% sucrose laurate, no outgrowth was observed after 5 days of incubation (data not shown).

Since the PA 3679 spores were very sensitive to such extremely low concentrations of sucrose laurate, no combinations of pressure and sucrose laurate were examined. Pressurization of *B. subtilis* 168 spores at 4,000 atm, 25°C for 30 min at pH 4.0 to 7.0 with subsequent plating on NA amended with 0.1 to 1.0% sucrose laurate, did not show any observable synergy (Figures 22 through 25). Similarly, spore samples that were pressurized in NB + sucrose laurate with the same combined treatments showed no decrease in CFU/mL as compared to non-pressurized samples (Figures 22 through 25). However, when the temperature during pressurization was increased to 45°C, a significant, reproducible, synergistic effect using mild heat, pressure and the sucrose laurate was observed. Pressurization of spores with an initial concentration of 1 x 10^6/mL for 15 min at pH 4.0, 5.0, or 6.0 were completely inhibited from outgrowth when plated on NA amended with as low as 0.1% sucrose laurate (Figures 26 through 28). The only pressure + sucrose laurate treatments that showed colony growth were those suspensions pressurized at pH 7.0 and plated in NA containing 0.1% sucrose laurate (<30 spores/mL remaining that represent reductions in CFU/mL of approximately 5 $\log_{10}$; Fig. 29). Exposure to higher concentrations of sucrose laurate (NA containing 0.25, 0.5 or 1.0% sucrose laurate) resulted in spore outgrowth of <10 CFU/mL (Fig. 29). When spore samples were pressurized in NB amended with sucrose laurate and then plated on unamended NA, no observable synergistic effect was observed (Figures 22 through 29), indicating that the continued presence of sucrose laurate is required to prevent spore outgrowth.



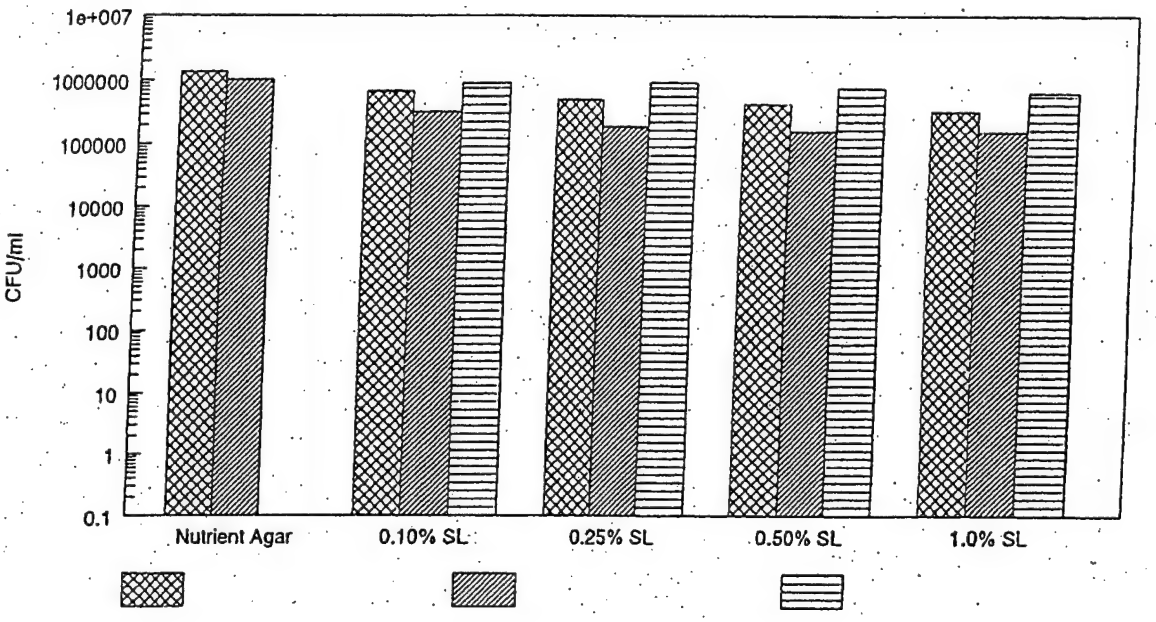
NA = Nutrient Agar; NB = Nutrient Broth; SL = Sucrose Laurate

Figure 22. Effect of pressurization at 4000 atm on the survival of spores of *B. subtilis* 168 incubated in buffer (pH 4.0) and NB (SL amended, 0.1 to 1%) at 25°C for 30 min and plated on nutrient agar amended with 0.1% to 1.0% sucrose laurate



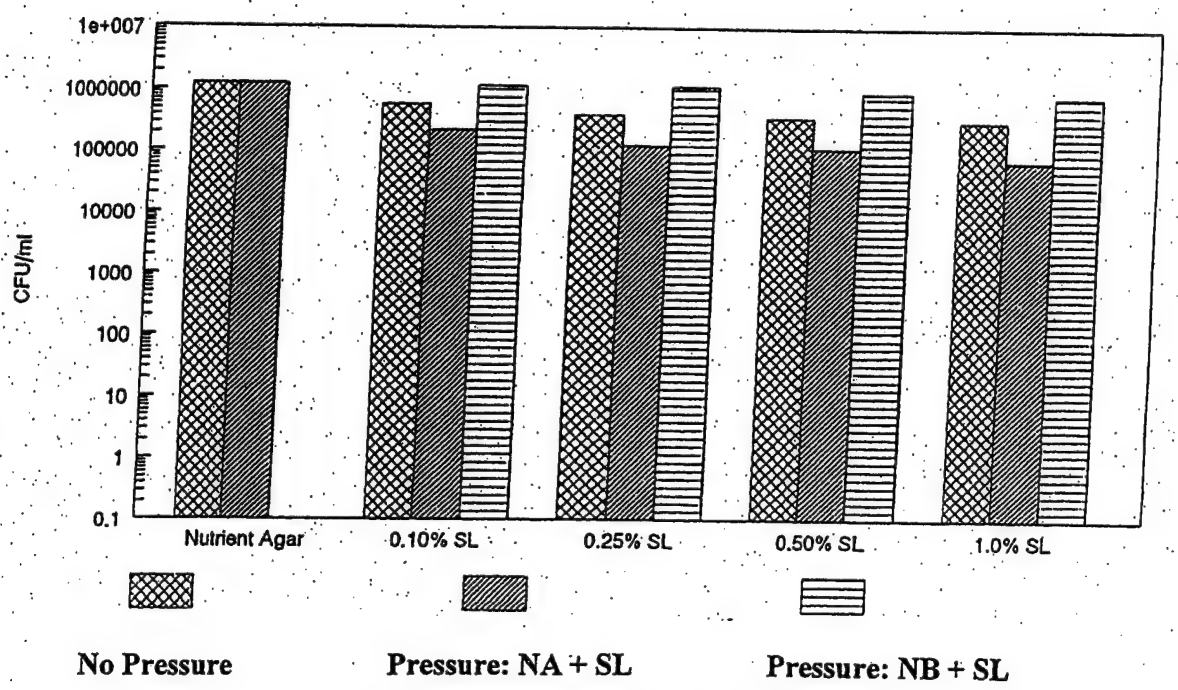
NA = Nutrient Agar; NB = Nutrient Broth; SL = Sucrose Laurate

Figure 23. Effect of pressurization at 4000 atm on the survival of spores of *B. subtilis* 168 incubated in buffer (pH 5.0) and NB (SL amended, 0.1 to 1%) at 25 °C for 30 min and plated on nutrient agar amended with 0.1% to 1.0% sucrose laurate



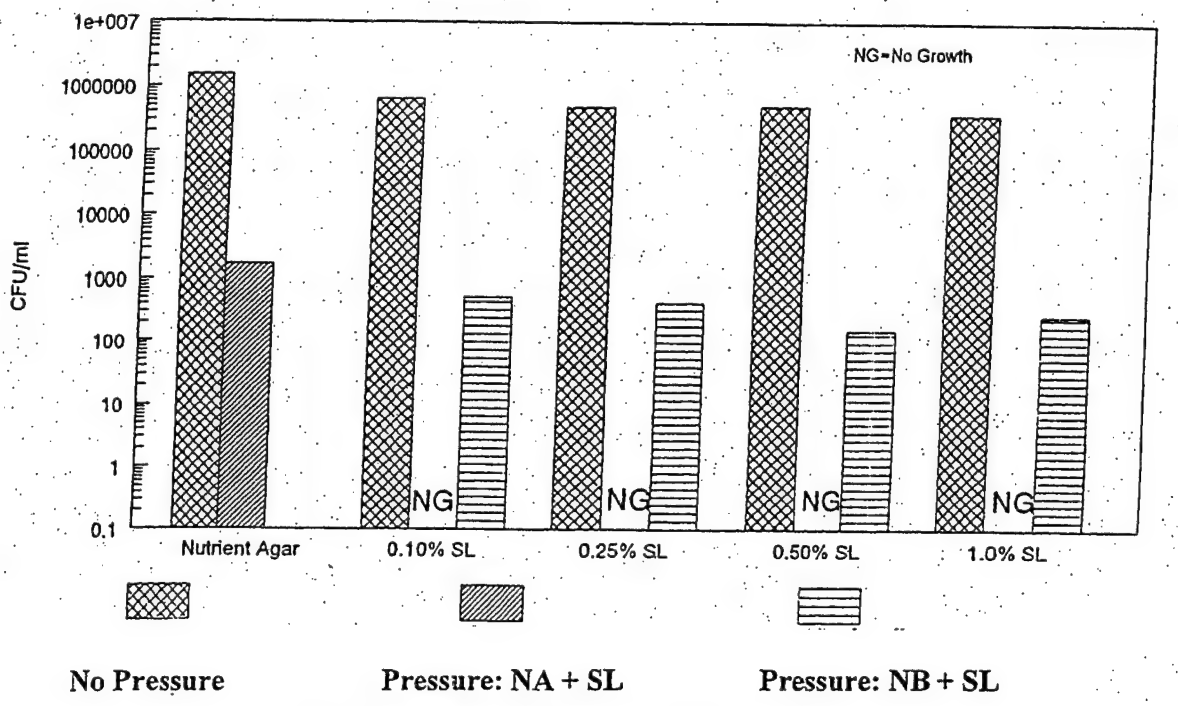
NA = Nutrient Agar; NB = Nutrient Broth; SL = Sucrose Laurate

Figure 24. Effect of pressurization at 4000 atm on the survival of spores of *B. subtilis* 168 incubated in buffer (pH 6.0) and NB (SL amended, 0.1 to 1%) at 25 °C for 30 min and plated on nutrient agar amended with 0.1% to 1.0% sucrose laurate.



NA = Nutrient Agar; NB = Nutrient Broth; SL = Sucrose Laurate

Figure 25. Effect of pressurization at 4000 atm on the survival of spores of *B. subtilis* 168 incubated in buffer (pH 7.0) and NB (SL amended, 0.1 to 1%) at 25 °C for 30 min and plated on nutrient agar amended with 0.1% to 1.0% sucrose laurate.



NA = Nutrient Agar; NB = Nutrient Broth; SL = Sucrose Laurate

Figure 26. Effect of pressurization at 4000 atm on the survival of spores of *B. subtilis* 168 incubated in buffer (pH 4.0) and NB (SL amended, 0.1 to 1%) at 45 °C for 15 min and plated on nutrient agar amended with 0.1% to 1.0% sucrose laurate.

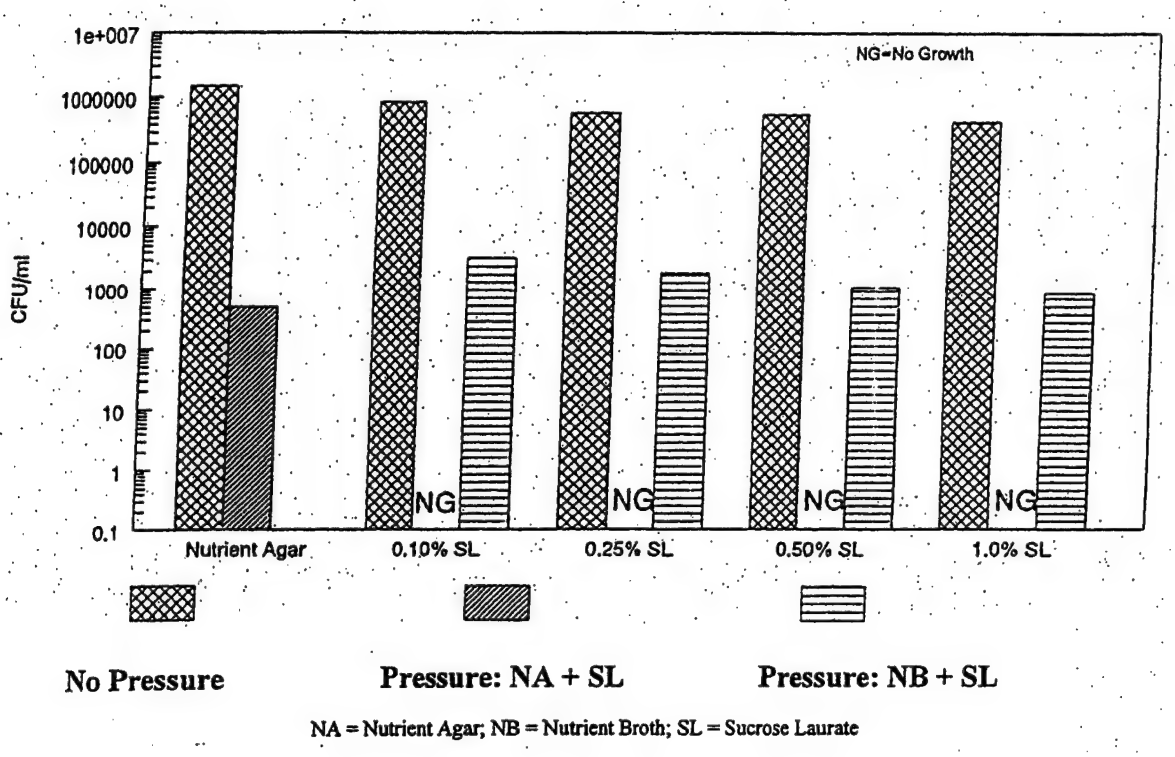


Figure 27. Effect of pressurization at 4000 atm on the survival of spores of *B. subtilis* 168 incubated in buffer (pH 5.0) and NB (SL amended, 0.1 to 1%) at 45 °C for 15 min and plated on nutrient agar amended with 0.1% to 1.0% sucrose laurate.

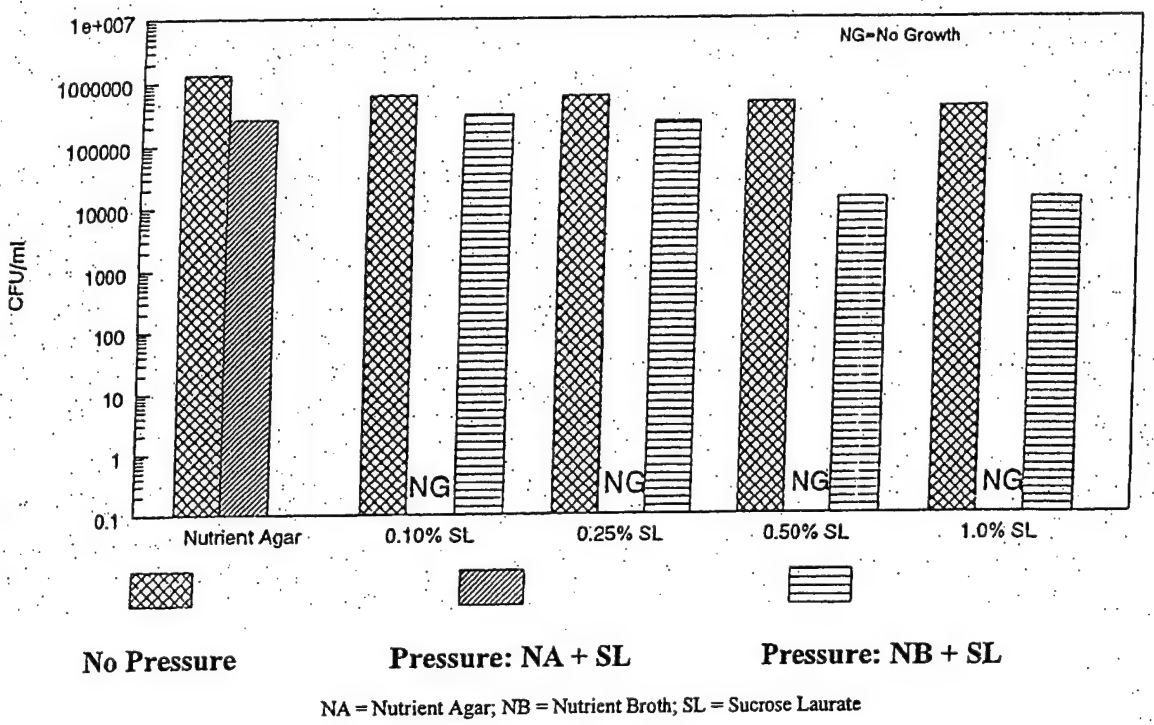


Figure 28. Effect of pressurization at 4000 atm on the survival of spores of *B. subtilis* 168 incubated in buffer (pH 6.0) and NB (SL amended, 0.1 to 1%) at 45 °C for 15 min and plated on nutrient agar amended with 0.1% to 1.0% sucrose laurate

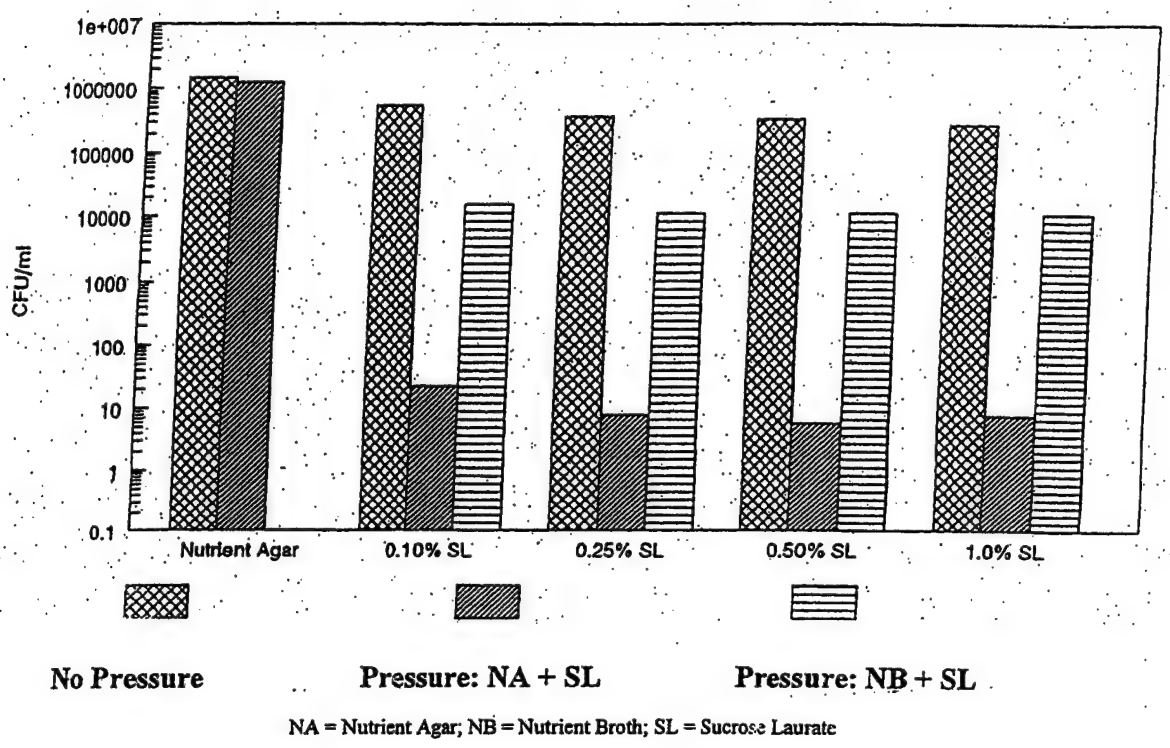


Figure 29. Effect of pressurization at 4000 atm on the survival of spores of *B. subtilis* 168 incubated in buffer (pH 7.0) and NB (SL amended, 0.1 to 1%) at 45 °C for 15 min and plated on nutrient agar amended with 0.1% to 1.0% sucrose laurate.

Inoculated pack studies with nisin

Inoculated pack studies without pressure treatment were done to determine a concentration of nisin that would allow outgrowth without pressurization so that nisin-pressure combinations could be studied (Table 1). Nisin levels of 5.0, 1.0 and 0.5 IU/mL were sufficient to prevent spore outgrowth for up to 14 d in the majority of pouches with suspensions of approximately 3 x 10^3 spores/pouch. When nisin concentrations were lowered to 0.1 and 0.05 IU/mL, all pouches (except 1 pouch in 10 for pH 6.0 and 0.1 IU/mL nisin combination) showed turbidity and gas production within 6 d. Therefore, these two nisin concentrations were chosen for combined use with pressurization, to see if pressurization of spores affected nisin sensitivity.

Fifty-pouch trials with initial concentrations of approximately 3 x 10^3 spores/pouch were conducted with treatments of 4,000 atm, pH 6.0 or 6.5 at 90 or 95°C for 15 min (Table 2). No pouches showed turbidity or swelling after 60 d incubation at 37°C. Pressurization at 70°C for 30 min, pH 6.0 or 6.5, gave 1 pouch in 50 turbid with gas production after incubation at 21 and 17 d, respectively. When the temperature range of pressure treatment was reduced from 90 - 95°C to 25 - 45°C for 30-min pressurizations at pH 6.0 or 6.5, all pouches were turbid with gas production within 4 d for all four treatment combinations.

Ten-pouch trials with initial concentrations of approximately 3 x 10^3 spores/pouch were done with combinations of pressure, nisin at two levels and moderate heat (Table 3). No pouches showed outgrowth after 60-d incubation at 37°C when treated at 4,000 atm, pH 6.0 or 6.5, with 0.5-IU/mL nisin at 55°C for 30 min. It is unclear why the higher concentration of nisin (0.1 IU/mL) displayed growth in several pouches. Treatments of 4,000 atm, with 0.1-IU/mL nisin at 45°C for 30 min at pH 6.0 or 6.5 had 8 out of 10 and 7 out of 10 pouches demonstrating turbidity and swelling after 5 d; at a

concentration of 0.05 IU/mL, pressure treatment at 45°C for 30 min was totally ineffective at either pH 6.0 or 6.5 where 10 out of 10 pouches failed after 5 d at 37°C (Table 3). All pouches were positive for outgrowth after 2 d at 37°C when treated for 30 min at 4,000 atm, pH 6.0 or 6.5, with either 0.1 or 0.05-IU/mL nisin at 25°C.

Fifty-pouch trials were conducted with spore inocula increased to approximately 3×10^4 and 3×10^5 spores/pouch with pressure treatment alone(Table 4 and 5). Treatments at 4,000 atm and 90°C for 15 min at pH 6.0 or 6.5 produced no pouches with turbidity or gas after 37°C incubation for 21 to 30 d at either spore concentration. Lowering the treatment temperature to 70°C for 30-min pressurizations of samples with 3×10^4 spores/pouch resulted in all pouches negative for outgrowth in pH 6.5 RCM (14-d incubation); however, at pH 6.0 (17-d incubation), 5 in 50 pouches were positive for turbidity and gas production. In all other parings of experiments matching pH 6.0 and pH 6.5 data, the slightly more acid pH was more antagonistic to outgrowth of spores of PA 3679.

Table 1. Inoculated pack study with *Clostridium sporogenes* PA 3679 (3×10^3 spores*/pouch) grown in reinforced clostridial medium (RCM) with combination treatments of pressure (0), time (10 and 15 min), nisin (0.0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 and 5.0 IU/mL), pH (6.0 and 6.5) and temperature (80 ° and 90 °C).

Temp. (C)	Time (min)	pH	Nisin (IU/ml)	# Turbid + Gas
90	15	6.5	0	50/50 (3 d)
90	15	6.0	0	50/50 (3 d)
80	10	6.5	5.0	0/10 (14 d)
80	10	6.0	5.0	1/10 (14 d)
80	10	6.5	1.0	1/10 (14 d)
80	10	6.0	1.0	1/10 (14 d)
80	10	6.5	0.5	4/10 (8 d)
80	10	6.0	0.5	1/11 (3 d)
80	10	6.5	0.4	9/10 (4 d)
80	10	6.0	0.4	7/10 (5 d)
80	10	6.5	0.3	9/10 (4 d)
80	10	6.0	0.3	10/10 (5 d)
80	10	6.5	0.2	
80	10	6.0	0.2	10/10 (5 d)
80	10	6.5	0.1	10/10 (2 d)
80	10	6.0	0.1	9/10 (6 d)
80	10	6.5	0.05	8/8 (3 d)
80	10	6.0	0.05	7/7 (3 d)

* 80 C for 10 min is typical heat-shock treatment parameters

Table 2. Inoculated pack study with PA 3679 (3×10^3 spores*/pouch) grown in RCM with combination treatments of pressure (4,000 atm), time (15 and 30 min), nisin (0), pH (6.0 and 6.5) and temperature (25 °, 45 °, 70 °, 85 °, 90 ° and 95 °C).

Temp. (C)	Time (min)	pH	Nisin (IU/ml)	# Turbid + Gas
95	15	6.5	0	0/48 (60 d)
95	15	6.0	0	0/48 (60 d)
90	15	6.5	0	0/52 (60 d)
90	15	6.0	0	0/50 (60 d)
85	30	6.5	0	0/10 (45 d)
85	30	6.0	0	0/10 (45 d)
70	30	6.5	0	1/50 (21 d)
70	30	6.0	0	1/50 (17 d)
45	30	6.5	0	10/10 (4 d)
45	30	6.0	0	10/10 (4 d)
25	30	6.5	0	10/10 (4 d)
25	30	6.0	0	10/10 (4 d)

Table 3. Inoculated pack study with PA 3679 (3×10^3 spores*/pouch) grown in RCM with combination treatments of pressure (4,000 atm), time (30 min), nisin (0.05 and 0.1 IU/mL), pH (6.0 and 6.5) and temperature (25 °, 45 ° and 55 °C).

Temp. (C)	Time (min)	pH	Nisin (IU/ml)	# Turbid + Gas
55	30	6.5	0.1	6/50 (60 d)
55	30	6.0	0.1	1/50 (60 d)
55	30	6.5	0.05	0/50 (60 d)
55	30	6.0	0.05	0/50 (60 d)
45	30	6.5	0.1	8/10 (5 d)
45	30	6.0	0.1	7/10 (5 d)
45	30	6.5	0.05	10/10 (5 d)
45	30	6.0	0.05	10/10 (5 d)
25	30	6.5	0.1	10/10 (2 d)
25	30	6.0	0.1	10/10 (2 d)
25	30	6.5	0.05	10/10 (2 d)
25	30	6.0	0.05	10/10 (2 d)

Table 4. Inoculated pack study with PA 3679 (3×10^3 spores*/pouch) grown in RCM with combination treatments of pressure (4,000 atm), time (15 and 30 min), nisin (0.0), pH (6.0 and 6.5) and temperature (55 °, 70 ° and 90 °C).

Temp. (C)	Time (min)	pH	Nisin (IU/ml)	# Turbid + Gas
90	15	6.5	0	0/50 (60 d)
90	15	6.0	0	0/50 (60 d)
70	30	6.5	0	0/50 (60 d)
70	30	6.0	0	5/50 (60 d)
55	30	6.5	0	17/50 (17 d)
55	30	6.0	0	0/50 (20 d)

Table 5. Inoculated pack study with PA 3679 (3×10^5 spores*/pouch) grown in RCM with combination treatments of pressure (4,000 atm), time (15 and 30 min), nisin (0.1 IU/mL), pH (6.0 and 6.5) and temperature (55 °, 70 ° and 90 °C).

Temp. (C)	Time (min)	pH	Nisin (IU/ml)	# Turbid + Gas
90	15	6.5	0	0/50 (60 d)
90	15	6.0	0	0/50 (60 d)
70	30	6.5	0	1/50 (60 d)
70	30	6.0	0	0/50 (60 d)
55	30	6.5	0	30/50 (14 d)
55	30	6.0	0	14/50 (14 d)
55	30	6.5	0.1	13/50 (13 d)
55	30	6.0	0.1	3/50 (60 d)

CONCLUSIONS

In this project, the combined and singular effects of exposure to hydrostatic pressure, nisin and other additives, elevated temperature, and acidic pH were examined for inactivation of spores of *Bacillus subtilis* 168 and *Clostridium sporogenes* PA 3679. PA3679 was more resistant to nisin, but generally proved to be more sensitive to exposure to pressure, heat, acidic pH and surcrose laurate. Data suggest several areas for development of process optimization of high hydrostatic pressure for inactivation of spores of *Bacillus* and *Clostridium* species.

Pressure oscillations at 45 °C/4,000 atm resulted in a greater inactivation of spores than a time-equivalent single pressure treatment (1×10^6 spores/mL), but only when each pressure pulse consisted of a 5 min exposure at 4,000 atm. When the pulse included the come-up time to full pressure (4,000 atm), the pressure oscillation was no longer superior to a time-equivalent single-pressure exposure (actual exposure to 4,000 atm approximately 1.5 to 2 min). pressure oscillation appears to be a processing parameter that requires careful optimization in any specific approach employing pressure.

It is well established that pH is a very important processing parameter for the application of pressure. This was confirmed with data provided in this study. The buffer type was a factor in the response of spores to pressure. Citrate-phosphate buffer was more detrimental to spore outgrowth after pressurization than the other buffers examined.

Spores of *B. subtilis* 168 and *C. sporogenes* PA 3679 differed in response to some common food preservatives (sodium acetate, sodium nitrite and benzoic acid); however, no enhancement or synergy of spore inactivation was evident with combinations of pressure treatment and these additives.

Treatment syngery was suggested with the food additive, sucrose laurate. Alone, sucrose laurate was an effective inhibitor of PA 3679, but the compound had little effect against *B. subtilis* 168. Pressure treatment at ambient temperature (25 °C) and sucrose laurate demonstrated little enhancement of antagonistic effect versus spores of *B. subtilis* 168. When pressure treatment was done at 45 °C, the level of spore inhibition exceeded the additive effects of pressure and sucrose laurate used alone. In the pH range of 4.0 to 6.0, no surviving spores were detectable from suspensions of 1×10^6 spores/mL after pressurization at 45 °C. For this reason, sucrose laurate had to remain in contact with the spores.

Since the food industry commonly uses inoculated pack studies to examine new approaches in food process development, this format was used in process optimization of pressure, nisin and treatment temperature versus PA3679. Nisin levels of 0.5 to 5.0 IU/mL were sufficient to prevent spore outgrowth for two weeks of storage at 37 °C with spore suspensions of 3×10^3/pouch incorporating a 4,000 atm pressure treatment temperature of 80 °C. Higher spore loads negated the preservative action of nisin and required an increase in other treatment parameters.

The results from this project indicate that high hydrostatic pressure can be used as an effective processing method to control the outgrowth of bacterial endospores. If maximum pressure in the range of 4,000 atm are to be used, other processes or additives must be employed. As established in other areas of food science and technology, manipulation of product pH, use of food additives, selection of process temperatures, and proper packaging and storage are parameters that must be understood and used properly in order for pressure technology to be effective in foods that are susceptible to outgrowth of bacterial endospores (e.g., the hurdle effect).

This document reports research undertaken at the U.S. Army Soldier and Biological Chemical Command, Soldier Systems Center, Natick, MA, and has been assigned No. NATICK/TR-02/025 in a series of reports approved for publication.

REFERENCES

Farkas, J. 1994. Tolerance of spores to ionizing radiation: Mechanisms of inactivation, injury, and repair. J. Appl. Bacteriol. **76**:81S-90S.

Funtenberger, S., E. Dumay and J.C. Cheftel. 1995. Pressure-induced aggregation of β-lactoglobulin in pH 7.0 buffers. Lebensm.-Wiss. u.-Technol. **28**:410-418.

Giddings, N.J., A.H. Allard and B.H. Hite. 1929. Inactivation of the tobacco-mosaic virus by high pressures. Phytopathology **19**:749-750.

Haas, G.J., H.E. Prescott Jr., E. Dudley, R. Dik, C. Hintlian and L. Keane. 1989. Inactivation of microorganisms by carbon dioxide under pressure. J. Food Safety **9**:253-265.

Hite, B.H. 1899. The effect of pressure in the preservation of milk. W. Va. Univ. Agr. Exp. Stn. Bull. (Morgantown) **58**:15-35.

Hoover, D.G., C. Metrick, A.M. Papineau, D.F. Farkas and D. Knorr. 1989. Biological effects of high hydrostatic pressure on food microorganisms. Food Technol. **43**:99-107.

Jay, J. 1986. Food preservation. In *Modern Food Microbiology*. Van Nostrand Reinhold Co., New York. pp. 257-296.

Johnson, F.H. and E.E. ZoBell. 1949. The retardation and thermal disinfection of *Bacillus subtilis* spores by hydrostatic pressure. J. Bacteriol. **57**:353-358.

Kalchayanand, N., A. Sikes, C.P. Dunne and B. Ray. 1994. Hydrostatic pressure and electroporation have increased bacteriocidal efficiency in combination with bacteriocins. Appl. Environ. Microbiol. **60**:4174-4177.

Leistner, U.W. and W. Rodel. 1976. In *Intermediate Moisture Foods*. ed. Davies, R., G.G. Birch and K.J. Parker, London: Applied Science Publishers.

Mallidis, C.G. and D. Drizou. 1991. Effect of simultaneous application of heat and pressure on the survival of bacterial spores. J. Appl. Bacteriol. **71**:285-288.

Mertens, B. and D. Knorr. 1992. Developments of nonthermal processes for food preservation. Food Technol. **46**:156-161.

Nicholson, W.l. and P. Setlow. 1990. Sporulation, germination and outgrowth. In *Molecular Biological Methods for Bacillus*, C.R. Hardwood and S.M. Cutting, eds. pp. 391-450, John Wiley and Sons, Chichester, England.

Ogawa, H., K. Fukuhisa, Y. Kubo and H. Fukumoto. 1990. Pressure inactivation of yeasts, molds, and pectinesterase in Satsuma mandarin juice: Effects of juice concentration, pH, organic acids, and comparison with heat sanitation. Agr. Biol. Chem. **54**:1219-1225.

Sale, J.H., G.W. Gould and W.A. Hamilton. 1970. Inactivation of bacterial spores by hydrostatic pressure. J. Gen. Microbiol. **60**:323-334.

Setlow, P. 1994. Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. J. Appl. Bacteriol. **76**:49S-60S.

Shigehisa, T., T. Ohmori, A. Saito, S. Taji and R. Hayashi. 1991. Effects of high hydrostatic pressure of characteristics of pork slurries and inactivation of microorganisms associated with meat and meat products. Int. J. Food Microbiol. **12**:207-216.

Sikes, A. and S. Whitfield. 1992. Antimicrobial activity of sucrose laurate, EDTA, and BHA alone and in combination. Proceedings Fourth Sci. Symp., Natick, MA. pp. 237-250.

Tanaka, T. and K. Hatanaka. 1992. Application of hydrostatic pressure to yoghurt to prevent its after-acidification. Nippon Shokuhin Kogyo Gakkaishi **39**:173-177.

Timson, W.J. and A.J. Short. 1965. Resistance of microorganisms to hydrostatic pressure. Biotechnol. Bioengineering 7:139-159.